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OPTIMIZATION OF HYDROLYSIS AT DETERMINATION OF AMINO ACID CONTENT IN FOOD AND FEED PRODUCTS

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The authors determined the amino acid composition of maize, soybeans, milk powder and meat meal using the 6 *M* HCl, the 3 *M* para-toluenesulphonic acid and the 3 *M* mercaptoethanesulphonic acid methods of protein hydrolysis, and studied the decomposition of the individual amino acids due to the effect of hydrolysis and the processing of the hydrolysate. It was found that hydrolysis with 6 *M* HCl at 105 °C for 24 hours, followed by neutralization with 4 *M* sodium hydroxide gave the optimum result for all amino acids with the exception of tryptophan. Under acidic conditions tryptophan is almost entirely decomposed, while 20–50% of the cysteine is decomposed, depending on the quality of the material under examination, compared with the quantity determined in the form of cysteic acid after oxidation with performic acid. Due to the proven loss of cysteine the authors elaborated a rapid, accurate method for determining the cysteine content of feed and food products. The method consists of oxidation with performic acid, followed by hydrolysis with 6 *M* HCl, after which 5–8 samples are fed onto the ion exchange column of the amino acid analyser at 2–3 minute intervals. This method allows the precision of the cysteine determination to reach the level obtained for the other amino acids. For tryptophan, results suitable for evaluation were only obtained using the sulphonic acid methods.

A 30% higher value was found for the tryptophan content after hydrolysis with mercaptoethanesulphonic acid than after hydrolysis with para-toluenesulphonic acid.

In the course of their examination the authors used cysteic acid as an internal standard, which had the effect of eliminating the reduction in colour intensity caused by the decomposition of ninhydrin.

A description is given of how the instrument can be calibrated for cysteic acid, and the results achieved after reference to an internal cysteic acid standard are compared to those obtained using the traditional standard calibration. All the analyses were carried out using an LKB-4011 automatic amino acid analyser.

Keywords: amino acids, protein hydrolysis, analysis of amino acids, protein in food and feed

When determining the amino acid composition of feed and food products the following errors are the most frequent:

- sampling errors;
- errors in hydrolysing the sample;
- instrumental errors committed during the analysis of the sample;
- errors in interpreting the chromatograms.

The report by Moore and Stein (1951) on the development of a method of analysing amino acids using ion exchange column chromatography gave

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rise to such intensive research efforts that, in the course of the last 30 years, the chromatography technique has been virtually perfected, the time required for chromatography reduced to a fraction of the original, and the minimum quantity of amino acid which can be demonstrated reduced by several orders of magnitude. The errors in measuring, committed in the course of sample analysis, have thus been reduced to a minimum. Provided the samples taken in the required manner and the chromatograms are interpreted very carefully, or with the help of integrators, the errors committed in the course of amino acid analysis can be limited to those due to errors in hydrolysing the sample. Adequate sampling, perfect chromatographic technique and correct interpretation are all in vain if errors have been committed during the hydrolysis.

In addition to the hydrolysis with 6 *M* HCl recommended by MOORE and STEIN (1963) a number of other methods have been elaborated for protein hydrolysis. The most important of these are perhaps the 3 *M* para-toluenesulphonic acid method proposed by LIU and CHANG (1971) and the mercaptoethanesulphonic acid method used by PENKE and co-workers (1974), developed by the authors in order to determine the tryptophan content of proteins.

In materials with low protein contents (certain feed and food products), which contain little cysteine and cystine, the traditional procedure for determining cysteine is inaccurate and unreliable compared to the other amino acids for the following reasons: It is difficult to evaluate the tiny, flat cysteine peak without an integrator and there is a great risk of subjective errors. If the quantity of cysteine is increased by applying a sample with a higher concentration to the ion exchange column, apart from cysteine all the other amino acids are impossible to evaluate and the presence of alanine and valine at too high a concentration disturbs the evaluation of cysteine.

A significant loss in cysteine content may occur during hydrolysis due to decomposition and oxidation. The decomposition of sulphur-containing amino acids was first demonstrated by MARTIN and SYNGE (1945), while YORITAKA and ONO (1954) also showed that cysteine may decompose into alanine, serine and glycine in the course of hydrolysis. According to OSONO and co-workers (1955) it can also decompose into homocysteine, homocystine and glycine. SCHRAM and co-workers (1954) recommended oxidation with performic acid for the determination of sulphur-containing amino acids. This method was modified by MOORE and STEIN (1963) in such a way that it could be routinely applied. In determining the amino acid composition of ribonuclease HIRS (1956) was able to determine the sulphur-containing amino acids with great precision after performic acid oxidation in the form of methioninesulphonic acid or cysteic acid. Important work was done in this field by DOVE and FRENEY (1979) who examined the effect of oxidation and hydrolysis on the composition of milk powder. A detailed examination was made of the circumstances of performic acid oxidation, including over-oxidation and how it can be

avoided. It was found that when hydrolysis was carried out in a vacuum, with the complete exclusion of air, there was no substantial difference in the methionine determined from the oxidized or non-oxidized samples, whereas 40% less cysteine was found in the non-oxidized form than after determination in the form of cysteic acid following oxidation with performic acid.

For amino acids the ninhydrin reagent which produces the colour reaction is extremely sensitive to light and to the oxygen content of the air, so the colour intensity produced in the presence of amino acids changes as a function of time. The frequent running of a calibration standard can be avoided by using an internal standard, which acts as a reference for all the amino acids, following the changes in the intensity of the colour reaction produced with ninhydrin, since the analysis of the internal standard, of a known concentration, is carried out parallel to that of the other amino acids. The internal standard generally used is norleucine, the application of which was reported by HEESE and co-workers (1972). In amino acid analysers or buffer systems where the separation of isoleucine, leucine and norleucine is not complete, norleucine cannot be used as the internal standard, because it makes the separation of isoleucine and leucine even more difficult and appears on the chromatogram as a twin peak with isoleucine, making evaluation very unreliable. It is impossible to refer the amino acids to the area of an amino acid peak which is difficult to interpret.

The majority of authors determined the amino acid composition of pure peptides or purified proteins and their methods have either not been tested on materials with a high carbohydrate content or the tests did not give satisfactory results. In Hungary there is neither a standard nor a recommendation for the determination of the amino acid composition of feed and food products. The results obtained in amino acid analysers operating in various laboratories throughout the country can only be compared if the determination is carried out using identical methods. The current experiments were aimed at selecting from among the numerous methods of hydrolysis reported in the literature those which appear the most suitable for examining feed and food products in a laboratory with a medium range of equipment.

By applying various methods of hydrolysis it was hoped to determine the extent to which the temperature of hydrolysis, the processing of the hydrolysate and the use of sulphonic acids influence the amino acid composition of samples with different protein contents, with particular regard to methionine, cysteine and tryptophan. In addition to the methods of hydrolysis mentioned above the rapid method elaborated by CSAPÓ and WÖLLER (1980) and CSAPÓ (1982) was also used to determine the cysteine content of the samples in the form of cysteic acid after oxidation with performic acid. Due to the difficulties encountered in separation when using norleucine as the internal standard, the results of the analysis were evaluated both with the traditional standard

calibration and with reference to an internal standard consisting of cysteic acid. The results were statistically analysed and a test of significance was carried out to demonstrate the similarities and differences. The means, standard deviation and the comparison of the means (t-test) were calculated using an HT PTK-1050 pocket computer. The single factor analysis of variance and the testing of the homogeneity of variance (Bartlett test) were carried out using an HT PTK-1096 pocket computer. The calculated χ^2 value was smaller in every case than the χ^2 value given in the table at $P = 1\%$. The standard deviation of data submitted to variance analysis and the significance test therefore do not differ significantly from each other.

1. Materials and methods

1.1. Materials tested

In order that the investigation should cover samples with both low and high protein contents, the methods described below were used to determine the amino acid composition of freely marketed maize (crude protein content, hereafter cr. prot. = 9.5%), of ground soybeans marketed by Agrocomplex (cr. prot. = 48.6%), of freely marketed meat meal (cr. prot. = 59.9%) and of a milk compound dried at −50 °C by lyophilization, originating from the Experimental Station of the Kaposvár College of Agriculture (cr. prot. of the milk powder = 35.4%). The crude protein content of the samples was determined using a Kjel-Foss 16 200 rapid nitrogen analyser (in the cases of milk powder and meat meal the crude protein content was calculated from the nitrogen % using a conversion factor of 6.38, while in the case of maize and soybeans the conversion factor was 6.25).

1.2. Hydrolysis and the processing of the hydrolysate

After a preliminary removal of fat, 100 mg of each sample, irrespective of crude protein content, was measured into a 10 cm^3 medical ampoule previously washed with chromated sulphuric acid. In all four samples the protein was hydrolysed using the following methods:

1.2.1. Hydrolysis with 6 M *HCl for 24 hours at 105 °C* (method 1). Two cm^3 6 *M* HCl was added to the sample in the ampoule, after which it was brought into a state of suspension and left to stand for half an hour. After the addition of one drop (approx. 40–50 mg) of phenol, sample particles stuck to the wall of the ampoule were washed down with 8 cm^3 6 *M* HCl, then, after passing nitrogen gas through the samples for 5 minutes the ampoules were sealed in a gas flame. After hydrolysis for 24 hours at 105 ±3 °C, the ampoules were cooled,

broken open and the pH value adjusted to 2.2 using 4 *M* sodium hydroxide. The whole material was then washed into a normal 25 cm^3 flask with pH 2.2 citrate buffer. The sample was filtered through Filtrak 388 filter paper and then stored in teflon vessels in a deep-freezer at −25 °C until they were applied to the amino acid analyser.

1.2.2. Hydrolysis with 6 M *HCl for 24 hours at 90 ±3 °C* (method 2). With the exception of the temperature the method is identical with the first method.

1.2.3. Hydrolysis with 6 M *HCl for 24 hours at 120 ±3 °C* (method 3). With the exception of the temperature the method is identical with the first method.

1.2.4. Hydrolysis with 3 M *p-toluenesulphonic acid for 24 hours at 105 ±3 °C* (method 4). The hydrolysis with 3 *M* p-toluenesulphonic acid (containing 0.2% tryptamine 3-(2-aminoethyl) indole) was adapted from the method of LIU and CHANG (1971) to suit the laboratory facilities available. The preparation and processing of the sample are identical with that in the first method.

1.2.5. Hydrolysis with 3 M *mercaptoethanesulphonic acid for 24 hours at 105 ±3 °C* (method 5). The method of PENKE and co-workers (1974) was adapted to the laboratory facilities available. The preparation and processing of the sample were identical with that in the first method.

1.2.6. Hydrolysis with 6 M *HCl for 24 hours at 105 ±3* °C (method 6). The hydrolysis is the same as that described in method 1, while the processing of the sample was conducted as follows. The cooled ampoules were broken and the contents washed into 100 cm^3 round flasks using bi-distilled water, after which they were evaporated to dryness in an atmosphere of nitrogen in a rapid rotational evaporator. The residue was dissolved in 2×10 cm^3 bi-distilled water and again evaporated to dryness. The residue was dissolved in pH 2.2 citrate buffer filtered through Filtrak 388 filter paper and stored in a deep-freezer at −25 °C until application to the amino acid analyser.

1.2.7. Hydrolysis with 6 M *HCl for 24 hours at 105 ±3 °C* (method 7). Processing was carried out in the same manner as in method 6, except that the evaporation in the rapid rotational evaporator took place in an atmosphere of air instead of nitrogen.

1.2.8. Performic acid oxidation after HIRS *(1956).* The protein hydrolysis was carried out as in method 1. using 6 *M* HCl.

1.3. Analysis

The amino acid content of the samples was determined using an LKB 4101 automatic amino acid analyser with a Merck amino acid calibration standard and a Merck cysteic acid internal standard. After suitable dilution a quantity of sample corresponding to approx. 50 n*M*, amino acid was applied to the ion exchange column, which meant that for maize the original stock solution was diluted five times and for meat meal thirty-five times.

Dimensions of the ion exchange

column: 500×6 mm;
ion exchange resin: CHROMEX UA-8;
buffer flow rate: 60 cm^3 per h;
ninhydrin flow rate: 30 cm^3 per h;
column temperature: 50 °C for 60 min, then
70 °C until the end of the analysis;
buffer A: pH = 3.25; Na molarity = 0.2; 25 min;
buffer B: pH = 4.25; Na molarity = 0.2; 60 min;
buffer C: pH = 6.35; Na molarity = 1.2; 55 min;
sodium hydroxide: 0.4 *M*; 15 min;
equilibration: buffer A; 60 min.

The cysteine content of the samples was determined using buffer A and the tryptophan content using a pH = 6.0, Na molarity = 1.5 buffer, the other parameters being the same as those described above.

1.4. Evaluation of the chromatograms

The quantities of the various amino acids in the samples were evaluated by comparing the area under the peak on the chromatogram with the area under the peak given for an amino acid standard of known concentration. The area was calculated by multiplying the height of the peak by the width of the peak at half its height. The chromatograms were also evaluated by reference to a cysteic acid internal standard. Cysteic acid has all the characteristics of an internal standard:

- it is an artificial product that does not occur in nature;
- it produces intensive colouring with ninhydrin at 570 nm;
- it can be easily separated from other amino acids and does not disturb the separation of the other amino acids;
- as the final oxidation product of cysteine it is an extremely stable compound.

The instrument was calibrated to cysteic acid by running a mixture of 10 standard amino acids. For each amino acid the cysteic acid equivalent proportionality constant (CAEPC) was calculated from the concentration of the standard solution, the quantity applied, the molecular mass and the area under the peak with the aid of the following equation:

$$\mathrm{CAEPC} = \frac{A_{\mathrm{Cys-SO_3H}} \times MM_{\mathrm{Cys-SO_3H}} \times \mathrm{mg_{AA}}}{A_{\mathrm{AA}} \times MM_{\mathrm{AA}} \times \mathrm{mg_{Cys-SO_3H}}}$$

Table 1

Amino acid composition of lyophilized milk powder (g amino acid per 100 g milk powder)

Amino acid	Sign of hydrolysis method													
	1		2		3		4		5		6		7	
	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$
Lysine	2.63	0.141	1.87	0.099	2.79	0.086	1.77	0.151	1.86	0.102	2.46	1.120	2.62	0.t68
Methionine	0.94	0.051	0.61	0.045	0.74	0.041	0.60	0.054	0.63	0.037	0.88	0.054	0.72	0.022
Cysteine	0.18	0.008	0.18	0.016	0.12	0.008	0.14	0.007	—		0.17	0.014	0.14	0.008
Valine	1.64	0.054	0.61	0.041	1.71	0.065	0.61	0.022	0.66	0.014	1.30	0.013	1.66	0.041
Isoleucine	1.20	0.086	0.50	0.022	1.70	0.136	0.61	0.067	0.86	0.036	1.02	0.065	1.05	0.059
Leucine	2.92	0.102	1.90	0.037	2.93	0.057	1.89	0.115	2.08	0.057	2.75	0.049	2.80	0.037
Tryptophan	—		—		—		0.23	0.016	0.29	0.014	—		—	
Total amino acids	32.11	0.376	22.85	0.670	33.52	0.370	23.15	0.315	24.08	0.765	32.38	0.679	32.22	0.593

Sign — Method of hydrolysis
1 — 6 *M* HCl, 105 °C, 24 hours, neutralized
2 — 6 *M* HCl, 90 °C, 24 hours, neutralized
3 — 6 *M* HCl, 120 °C, 24 hours, neutralized
4 — 3 *M* p-toluenesulphonic acid, 105 °C, 24 hours, neutralized
5 — 3 *M* mercaptoethanesulphonic acid, 105 °C, 24 hours, neutralized
6 — 6 *M* HCl, 105 °C, 24 hours, evaporation in an atmosphere of nitrogen
7 — 6 *M* HCl, 105 °C, 24 hours, evaporation in an atmosphere of air
Crude protein content (N % × 6.38) of the lyophilized milk powder = 35.4%, dry matter content = 91.0%
Number of measurements (n) = 3

Results of the homogeneity test on variance (P = 1%)

Amino acid	Degree of freedom	$\chi^2_{calc.}$	$\chi^2_{tabl.}$
Lysine	6	1.20	16.8
Methionine	6	1.58	16.8
Cysteine	5	2.18	15.1
Valine	6	6.67	16.8
Isoleucine	6	5.94	16.8
Leucine	6	4.26	16.8
Tryptophan	1	0.03	6.6
Total amino acids	6	2.33	16.8

where

A_{Cys-SO_3H} = area under the cysteic acid peak,
MM_{Cys-SO_3H} = molecular mass of cysteic acid,
A_{AA} = area under the peak for the investigated amino acid,
MM_{AA} = molecular mass of the amino acid.

The quantity of the unknown amino acid was calculated from this equation after conversion to mg_{AA}.

2. Results

On analysing the amino acid compositions obtained for maize, milk powder, soybeans and meat meal after various types of hydrolysis, it was observed that for all four materials and for each amino acid the change caused by the method of hydrolysis was identical, so the changes can be traced from the results of the amino acid composition for one of the materials (milk powder).

2.1. Amino acid composition of milk powder

The amino acid compositions obtained for milk powder with various methods of hydrolysis are presented in Table 1.

The amino acids contained in the Table are those which showed the greatest changes as the result of the method of hydrolysis. A rise in the temperature of hydrolysis increases the amount of valine, isoleucine and leucine which can be demonstrated in the sample, while significantly reducing the cysteine and methionine contents. The sulphur-containing amino acids, methionine and cysteine are the most sensitive to evaporation in air or nitrogen, while tryptophan decomposes almost entirely as the result of hydrolysis in

Table 2

One way variance analysis on the results obtained using various methods of hydrolysis for the amino acid composition of milk powder

Amino acid	Degrees of freedom		F value calculated	F value tabulated	Level of significance
	numerator	denominator			
Lysine	6	14	23.533	7.43	***
Methionine	6	14	18.100	7.43	***
Cysteine	5	12	10.134	8.89	***
Valine	6	14	380.047	7.43	***
Isoleucine	6	14	55.70	7.43	***
Leucine	6	14	93.075	7.43	***
Total amino acids	6	14	153.633	7.43	***

***: $P \leq 0.1\%$

Table 3

Comparison of the results obtained using various methods of hydrolysis (1–7) for the lysine and methionine content of milk powder with the help of the t*-test*

		Lysine						
		1	2	3	4	5	6	7
	1	—	7.64 **	1.68 Ø	7.21 **	7.66 **	1.59 Ø	0.08 Ø
	2	8.40 **	—	12.15 ***	0.96 Ø	0.12 Ø	6.57 **	6.66 **
	3	5.29 **	3.70 *	—	10.16 ***	12.07 ***	3.87 *	1.56 Ø
Methionine	4	7.93 **	0.25 Ø	3.58 *	—	0.86 Ø	6.20 **	6.52 **
	5	8.24 **	0.59 Ø	3.45 *	0.79 Ø	—	6.60 **	6.70 **
	6	1.40 Ø	6.65 **	3.58 *	6.35 **	6.61 **	—	1.34 Ø
	7	6.86 **	3.80 *	0.74 Ø	3.56 *	3.62 *	4.75 **	—

Ø: $P \geq 5\%$; *: $P \leq 5\%$; **: $P \leq 1\%$; ***: $P \leq 0.1\%$.

Table 4

Comparison of the results obtained by various methods of hydrolysis (1–7) for the valine and isoleucine content of milk powder using the t*-test*

		Valine						
		1	2	4	3	5	6	7
	1	—	26.31 ***	1.43 Ø	30.60 ***	30.43 ***	10.60 ***	0.51 Ø
	2	13.66 ***	—	24.79 ***	0 Ø	2.00 Ø	27.78 ***	31.36 ***
	3	5.38 **	15.09 ***	—	27.76 ***	27.35 ***	10.71 ***	1.12 Ø
Isoleucine	4	9.37 ***	2.70 Ø	12.45 ***	—	3.32 *	5.42 **	39.09 ***
	5	6.32 **	14.78 ***	10.34 ***	5.69 **	—	58.02 ***	39.98 ***
	6	2.89 *	13.13 ***	7.81 **	7.61 **	3.73 *	—	4.57 **
	7	2.49 Ø	15.13 ***	7.59 **	8.54 **	4.76 *	0.59 Ø	—

For legend see Table 3

Table 5

Comparison of results obtained by various methods of hydrolysis (1–7) for the leucine content and the total amino acids of milk powder using the t*-test*

		Leucine						
		1	2	3	4	5	6	7
Total amino acids	1	—	16.28 ***	0.15 Ø	11.71 ***	12.45 ***	2.60 Ø	1.92 Ø
	2	20.88 ***	—	26.25 ***	0.14 Ø	4.59 *	23.98 ***	29 79 ***
	3	4.60 *	24.15 ***	—	14.03 ***	18.26 ***	4.15 *	3 31 *
	4	31.64 ***	0.70 Ø	36.96 ***	—	2.56 Ø	11.92 ***	13.05 ***
	5	16.32 ***	2.09 Ø	19.24 ***	1.95 Ø	—	15.44 ***	18.35 ***
	6	0.17 Ø	17.12 ***	2.78 *	21.13 ***	8.20 **	—	1.41 Ø
	7	0.27 Ø	18.14 ***	3.22 *	23.40 ***	14.57 ***	0.12 Ø	—

For legend see Table 3

hydrochloric acid and can only be determined after hydrolysis with sulphonic acid. The results of one way variance analysis on the results, and those of the significance tests are presented in Tables 2, 3, 4 and 5.

2.2. The cysteine content of maize, milk powder, soybeans and meat meal determined using the experimental methods and by accelerated application after performic acid oxidation

The cysteine contents of maize, milk powder, soybeans and meat meal, measured using various methods, are contained in Table 6.

The results of single factor analysis of variance are presented in Table 7, and the test of significance on the results obtained with various methods of hydrolysis for the cysteine content of the experimental materials can be seen in Tables 8 and 9.

2.3. Determination of the tryptophan content of the samples

The tryptophan content of maize, milk powder, soybeans and meat meal measured after various methods of hydrolysis and expressed as g amino acid per 100 g sample are presented in Table 10 together with a comparison of the results with the aid of the *t* test.

Table 6

Cysteine content of maize, milk powder, soybeans and meat meal measured using various methods of hydrolysis (g amino acid per 100 g sample)

Material	Sign of hydrolysis method													
	1		2		3		4		6		7		Oxidized	
	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$
Maize	0.10	0.008	0.09	0.005	0.04	0.005	0.07	0.005	0.14	0.008	0.09	0.005	0.17	0.008
Milk powder	0.18	0.016	0.18	0.008	0.12	0.008	0.14	0.014	0.17	0.008	0.14	0.008	0.30	0.014
Soybeans	0.56	0.014	0.34	0.014	0.52	0.014	0.34	0.022	0.54	0.022	0.32	0.016	0.88	0.037
Meat meal	0.80	0.037	0.49	0.024	0.59	0.022	0.58	0.016	0.66	0.029	0.52	0.018	0.92	0.037

Sign — Method of hydrolysis
1 — 6 *M* HCl, 105 °C, 24 hours, neutralized
2 — 6 *M* HCl, 90 °C, 24 hours, neutralized
3 — 6 *M* HCl, 120 °C, 24 hours, neutralized
4 — 3 *M* p-toluenesulphonic acid, 105 °C, 24 hours, neutralized
6 — 6 *M* HCl, 105 °C, 24 hours, evaporated in an atmosphere of nitrogen
7 — 6 *M* HCl, 105 °C, 24 hours, evaporated in an atmosphere of air
Oxidized: Performic acid oxidation after the method of HIRS (1956)
Maize: cr. prot. = 9.5%, dry matter = 88%;
Milk powder: cr. prot. = 35.4%, dry matter = 91%;
Soybeans: cr. prot. = 48.6%, dry matter = 89%;
Meat meal: cr. prot. = 59.9%, dry matter = 90%.

Results of the homogeneity test on variance

Material	Degrees of freedom	$\chi^2_{calc.}$
Maize	6	1.28
Milk powder	6	2.17
Soybeans	6	3.14
Meat meal	6	2.11

Table 7

Single factor analysis of variance on the results obtained using various methods of hydrolysis for the cysteine content of maize, milk powder, soybeans and meat meal

Material	Degrees of freedom		F value calculated	F value tabulated	Level of significance
	numerator	denominator			
Maize	6	28	89.205	5.24	***
Milk powder	6	28	52.930	5.24	***
Soybeans	6	28	167.015	5.24	***
Meat meal	6	28	64.052	5.24	***

***: $P \leq 0.1\%$.

Table 8

Comparison of results obtained by various methods of hydrolysis (1–7) for the cysteine content of maize and milk powder using the t*-test*

		Maize						
		1	2	3	4	6	7	Oxidized form
Milk powder	1	—	1.84 Ø	11.02 ***	5.51 **	6.12 **	1.84 Ø	10.72 ***
	2	0 Ø	—	12.25 ***	4.90 **	9.18 ***	0 Ø	14.69 ***
	3	5.81 **	9.18 ***	—	7.34 **	18.36 ***	12.25 ***	23.87 ***
	4	3.26 *	4.30 *	2.15 Ø	—	12.85 ***	4.90 **	18.36 ***
	6	0.97 Ø	1.53 Ø	7.65 **	3.22 *	—	9.18 ***	4.60 **
	7	3.87 *	3.87 *	3.06 *	0 Ø	4.59 *	—	14.69 ***
	Oxidized form	9.78 ***	12.89 ***	19.34 ***	13.99 ***	13.96 ***	17.19 ***	—

For legend see Table 3

2.4. *Comparison of the results obtained using the cysteic acid internal standard and the data of traditional evaluation*

Since the trend in the data obtained by traditional evaluation and by applying the cysteic acid internal standard was the same for all four experimental materials, the presentation of data on the amino acid composition of milk powder will be sufficient for an evaluation of the results. Table 11 shows the lysine, methionine and valine contents of milk powder expressed in g amino acid per 100 g protein and the amino acid quantities obtained by converting the results to g amino acid per 100 g milk powder, evaluated by applying the traditional and the cysteic acid internal standards, as a function of the time since ninhydrin preparation. The hydrolysis and the processing of the hydrolysate were carried out according to method 1. The data in the table are the mean values of 3 analyses.

Table 9

Comparison of results obtained by various methods of hydrolysis for the cysteine content of soybeans and meat meal using the t*-test*

		Soybeans						
		1	2	3	4	6	7	Oxidized form
Meat meal	1	—	19.25 ***	3.49 *	14.61 ***	1.33 Ø	19.55 ***	14.01 ***
	2	12.17 ***	—	15.75 ***	0 Ø	13.28 ***	1.63 Ø	23.64 ***
	3	8.44 **	5.32 **	—	11.96 ***	1.33 Ø	16.29 ***	15.76 ***
	4	9.45 ***	5.40 **	0.64 Ø	—	11.13 ***	1.27 Ø	21.73 ***
	6	5.16 **	7.82 **	3.33 *	4.18 *	—	14.01 ***	13.68 ***
	7	11.79 ***	1.73 Ø	4.27 *	4.32 *	7.10 **	—	24.06 ***
	Oxidized form	3.97 *	16.89 ***	13.28 ***	14.61 ***	9.58 ***	16.84 ***	

For legend see Table 3

Table 10

Tryptophan content measured using various methods of hydrolysis for maize, milk powder, soybeans and meat meal (g amino acid per 100 g sample) and comparison of the results using the t*-test*

Material	Sign of hydrolysis method				t test values
	4		5		
	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	
Maize	0.06	0.004	0.09	0.005	8.12***
Milk powder	0.23	0.016	0.29	0.014	4.89**
Soybeans	0.31	0.016	0.44	0.014	10.59***
Meat meal	0.34	0.008	0.56	0.029	12.67***

Maize: cr. prot. = 9.5%; dry matter = 88%; milk powder: cr. prot. = 35.4%, dry matter = 91%; soybeans: cr. prot. = 48.6%; dry matter = 89%; meat meal: cr. prot. = = 59.9%, dry matter = 90%. Number of measurements (n) = 5.

Method 4: 3 *M* p-toluenesulphonic acid, 105 °C, 24 hours, neutralized. Method 5: 3 *M* mercaptoethanesulphonic acid, 105 °C, 24 hours, neutralized.

**: $P \leq 1\%$; ***: $P \leq 0.1\%$.

Results of homogeneity test on variance

Material	Degrees of freedom	$\chi^2_{calc.}$
Maize	1	0.079
Milk powder	1	0.028
Soybeans	1	0.028
Meat meal	1	2.14

Table 11

Total amino acids in lyophilized milk powder (g amino acid per 100 g milk powder) and the lysine, methionine und valine content of milk powder protein (g amino acid per 100 g protein) evaluated by standard calibration and using an internal cysteic acid standard

Amino acid	Time after preparation of ninhydrin (days)											
	0		1		2		3		4		5	
	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$
With standard calibration												
Lysine	7.9	0.047	7.9	0.047	8.0	0.047	7.9	0.082	7.8	0.082	8.0	0.047
Methionine	2.5	0.082	2.4	0.082	2.5	0.047	2.4	0.047	2.5	0.047	2.6	0.082
Valine	6.3	0.082	6.2	0.047	6.1	0.047	6.3	0.082	6.2	0.082	6.1	0.047
Total amount of amino acids	33.72	0.137	32.91	0.153	31.73	0.233	31.45	0.131	30.90	0.214	29.21	0.193
Evaluated with internal cysteic acid standard												
Lysine	7.8	0.082	7.9	0.082	7.8	0.047	7.9	0.047	8.0	0.047	7.8	0.082
Methionine	2.5	0.047	2.5	0.082	2.5	0.047	2.4	0.082	2.4	0.047	2.5	0.082
Valine	6.2	0.047	6.1	0.047	6.2	0.047	6.2	0.082	6.1	0.047	6.1	0.082
Total amount of amino acids	33.72	0.086	33.54	0.229	33.29	0.115	33.48	0.049	33.79	0.108	33.81	0.128

Results of homogeneity test on variance

Amino acid	Degrees of freedom	$\chi^2_{calc.}$
With standard calibration		
Lysine	5	1.49
Methionine	5	1.48
Valine	5	1.48
Total amino acids	5	0.96
With internal cysteic acid standard		
Lysine	5	1.48
Methionine	5	1.48
Valine	5	1.49
Total amino acids	5	4.05

The tabulated value of $\chi^2 = 15.1$

Table 12

Comparison of results obtained by various types of evaluation for the total amino acids of milk powder using the t*-test*

	Standard calibration						
	Time after preparation of ninhydrin (days)	0	1	2	3	4	5
	0	—	6.83 **	12.75 ***	20.74 ***	19.22 ***	33.01 ***
	1	1.27 Ø	—	7.33 **	12.55 ***	13.23 ***	26.02 ***
Cysteic acid	2	5.19 **	1.69 Ø	—	1.81 Ø	4.54 *	14.43 ***
internal	3	4.20 *	0.44 Ø	2.63 Ø	—	3.80 *	16.63 ***
standard	4	0.88 Ø	1.71 Ø	5.49 **	4.53 *	—	10.16 ***
	5	1.01 Ø	1.78 Ø	5.23 **	4.17 *	0.21 Ø	—

Ø: $P \geq 5\%$; *: $P \leq 5\%$; **: $P \leq 1\%$; ***: $P \leq 0.1\%$.

Table 13

Comparison of results obtained for the lysine, methionine and valine content of milk protein using various methods of evaluation with the help of the t*-test*

Calculated *t*-values		
Lysine	Methionine	Valine
0.85	0.21	0.90

The differences are not significant at $P \geq 5\%$ probability level

Table 13 contains the results of significance tests on the values obtained after various types of evaluation for the lysine, methionine and valine contents of milk protein, while Table 12 summarizes the results of significance tests on the amino acid quantities obtained after various types of evaluation as a function of the time since ninhydrin preparation.

3. Conclusions

It can be seen from the one way variance analysis of the results obtained for the amino acid composition of milk powder that the various methods of hydrolysis give significantly different results at the $P = 0.1\%$ level for all the amino acids. An individual comparison of the effects of the various methods of hydrolysis for different amino acids leads to the following conclusions:

For the lysine content of milk powder expressed in g amino acid per 100 g milk powder there is no significant difference between methods 1, 3, 6 and 7. This is also true of methods 2, 4 and 5, where again there is no difference between the values for lysine content. The lysine content obtained with methods 1, 3, 6 and 7 is significantly higher at the P = 0.1 and 1% levels than that measured using methods 2, 4 and 5.

The methionine content obtained with methods 1 and 6 is significantly higher at the P = 1–5% level than that obtained with methods 3 and 7, and significantly higher at the P = 1% level than the value found for methods 2, 4 and 5.

In the case of isoleucine the result obtained with method 3 was significantly higher at the P = 1% level than that measured with methods 1, 6 and 7, and significantly higher at the P = 0.1% level than the value obtained by methods 2, 4 and 5.

Higher values of valine and leucine are given by method 3 than by methods 1, 6 and 7, but the difference is only significant at the P = 5% level, or not significant at all (with the exception of the valine content using method 6).

In the case of valine and leucine methods 1, 3, 6 and 7 give significantly higher results at the P = 0.1% level than methods 2, 4 and 5, while for isoleucine the difference is significant at the P = 0.1 and 1% levels (except for the valine content for method 6).

The total amino acids is not significantly different for methods 1, 6 and 7, or for methods 2, 4 and 5. The total amino acids obtained by methods 1, 3, 6 and 7 is significantly higher at the P = 0.1% level than that found by methods 2, 4 and 5. The total amino acids obtained by method 3 is significantly higher at the P = 5% level than that found by methods 1, 6 and 7.

Single factor variance analysis of the cysteine content of maize, milk powder, soybeans and meat meal measured using various methods of hydrolysis indicates that the different methods of hydrolysis give different values of cysteine at the P = 0.1% level.

It can be seen from Table 6 that the cysteine values obtained for all four materials were about 25–50% higher when the determination was carried out in the oxidated form than in the cysteine form. Methods 1 and 6 gave the highest production of cysteine, while methods 2, 3, 4 and 7 only gave around 50% of the quantity determined in the form of cysteic acid. In the case of soybeans and milk powder the cysteine contents measured by methods 1 and 6 did not differ significantly from each other, while both methods gave significantly higher results at the P = 0.1 and 5% level than methods 2, 3, 4 and 7, with the exception of 5 cases.

Using method 5 it proved impossible to demonstrate the cysteine content due to the formation of a thioether bond between the sulphydryl group of mercaptoethanesulphonic acid and the sulfhydryl group of cysteine. The

determination of cysteine in the form of cysteic acid gave a significantly higher value at the P = 0.1% level than that obtained with any of the other methods (with two exceptions, where the difference was significant at the P = 5% and the P = 1% levels).

The data in Table 10 show that hydrolysis with 3 *M* mercaptoethanesulphonic acid (method 5) gave an approx. 30–35% higher value for tryptophan than method 4, using 3 *M* para-toluenesulphonic acid (0.2% tryptamine). In the case of maize, soybeans and meat meal the difference was significant at the P = 0.1% level, and for milk powder at the P = 1% level. In the course of hydrolysis with hydrochloric acid (methods 1, 2, 3, 6 and 7) the tryptophan content of the samples was almost entirely decomposed.

Table 11 shows the lysine, methionine and valine contents of milk protein and the total amino acids of milk powder evaluated in the traditional manner and with the use of a cysteic acid internal standard. It can be seen from the data that the two methods of evaluation give significantly identical results for the amino acid composition expressed as g amino acid per 100 g protein. In the case of traditional evaluation, assuming that the standard was run on the day the ninhydrin was prepared, the total amino acids and the quantity of the individual amino acids (g amino acid per 100 g milk powder) decreased as a function of the time since ninhydrin preparation, while in the case of a cysteic acid internal standard these values remained practically constant. When evaluating with the aid of a cysteic acid internal standard the amino acid totals obtained did not differ significantly from each other in the majority of cases as a function of the time since ninhydrin preparation. The total amino acids obtained with traditional evaluation was significantly lower at the P = 1% level after 24 hours, and from then on at the P = 0.1% level, than the value obtained immediately after preparation of the ninhydrin.

On the grounds of the above, the following conclusions can be drawn from the experiments:

— Hydrolysis with 6 *M* HCl at 90 ± 3 °C (method 2), with 3 *M* mercaptoethanesulphonic acid (method 5) and with 3 *M* para-toluenesulphonic acid (methods 1, 2, 4) gave only 65—80% values for the amino acids in all four basic materials compared to the values obtained after hydrolysis at 105 ± 3 °C or 120 ± 3 °C.

— In the case of hydrolysis with sulphonic acid at 105 ± 3 °C or 6 *M* HCl at 90 ± 3 °C the production of valine, isoleucine and leucine is extremely low, being approx. 55—60% of that obtained after hydrolysis with 6 *M* HCl at 105 or 120 ± 3 °C.

— With a rise in the temperature of hydrolysis there is a rise in the total quantity of amino acids and in the quantity of valine, isoleucine and leucine which can be demonstrated in the sample, but there is a sharp reduction in the quantity of cystine and a slight decrease in the methionine content.

— The sulphur-containing amino acid content of samples evaporated in an atmosphere of nitrogen (method 6) is approx. 15–20% higher than that of samples evaporated in air, and does not differ significantly from the sulphur-containing amino acid content of neutralized samples (method 1).

— The cysteine content of the samples was one and a half to two times higher when determined in the form of cysteic acid after performic acid oxidation than when determined in the form of cysteine.

— For tryptophan, results suitable for evaluation were only obtained by sulphonic acid methods. The mercaptoethanesulphonic acid method (5) gave a value approx. 30% higher than that obtained with p-toluenesulphonic acid.

— The cysteic acid internal standard is extremely useful in determining the amino acid composition of food and feed products. Losses caused by the decomposition of ninhydrin can be eliminated when using the cysteic acid internal standard.

It is obvious from the above that a complete, accurate determination of the amino acid composition cannot be carried out with a single hydrolysis. Tryptophan can only be determined using the sulphonic acid methods (of which the 3 *M* mercaptoethanesulphonic acid method is to be preferred), but these cannot be applied for any of the other amino acids.

No significant difference was found for hydrolysis with 6 *M* HCl at 105 ± 3 °C between the amino acid composition of samples evaporated after neutralization (method 1) or those evaporated in an atmosphere of nitrogen (method 6). Since neutralization with 4 *M* NaOH is much easier to perform than evaporation of the hydrochloric acid in an atmosphere of nitrogen, despite the fact that it increases the sodium ion concentration of the sample, neutralization is certainly more practicable for the rapid processing of a large number of samples. In the case of both evaporation in an atmosphere of nitrogen and of neutralization a considerable loss of cysteine can be expected compared to the cysteine content determined in the oxidized form. In confirmation of the data of DOVE and FRENEY (1979) it was found that while the cysteine loss may be as great as 40–50%, if hydrolysis and processing are carried out under optimum conditions the loss of methionine is negligible.

On the basis of the examinations the following methods of hydrolysis are recommended for determining the amino acid composition of feed and food products:

— For the determination of all the amino acids except cysteine and tryptophan, hydrolysis with 6 *M* HCl at 105 ± 3 °C (method 1). With this method the sulphur-containing amino acids only decompose to a slight extent, and the yield of valine, isoleucine and leucine is also satisfactory. The total yield of amino acids is highest for hydrolysis at 120 °C, but in this case there is a considerable decomposition of sulphur-containing amino acids.

— For the determination of cysteine content alone, performic acid oxidation as described by HIRS (1956) and, after processing, the accelerated application described by CSAPÓ and WÖLLER (1980) and modified for use on food and feed products by CSAPÓ (1982).

— For the determination of tryptophan content, the 3 *M* mercaptoethanesulphonic acid method elaborated by PENKE and co-workers (1974).

— In order to facilitate evaluation, and for the accurate measurement of the quantity of amino acids in 100 g feed, the use of a cysteic acid internal standard is recommended in all laboratories where the separation of isoleucine--leucine–norleucine is not perfect.

*

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ACTIVATION ENTHALPY IN *VICIA FABA* PROTEIN GELS

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The activation enthalpy ΔH of segment mobility in the network of *Vicia faba* protein gels was ascertained by mechanical stress at different temperatures. Therefore, the stress relaxation of these gels was measured as a function of temperature. The so-called shift factor (a_T) was determined by shifting the relaxation curves towards a temperature–invariant master curve. The activation enthalpy was calculated according to the Arrhenius equation with the shift factor. The acetylated and corresponding non-acetylated *Vicia faba* proteins were investigated.

Keywords: activation enthalpy, *Vicia faba*, protein gels, shift factor

In order to obtain further information on the physico-chemical characterization of *Vicia faba* protein (SCHMANDKE et al., 1977, 1981a, 1981b, 1981c 1982; SCHMANDKE & MAUNE, 1982; ANDERSSON et al., 1985) the activation enthalpy ΔH of *Vicia faba* protein gels in aqueous media was investigated.

1. Material and methods

Spray dried *Vicia faba* protein isolate, fractionated from *Vicia faba* flours by extracting with 0.01 *N* NaOH and by isoelectric precipitation after addition of 1 *N* HCl until a pH 4.0 was reached, has been acetylated at pH 8 with acetic anhydride as described previously (SCHMANDKE et al., 1977); we prepared these proteins with acetylation degrees of 45% (II) and 78% (III), respectively. The corresponding non-acetylated proteins (I) were obtained by the same procedure as mentioned for the acetylated products but without using acetic anhydride. The sample IV is a *Vicia faba* protein, isolated from *Vicia faba* flour by extraction at pH 8 with 0.37 *M* NaCl and subsequent isoelectric precipitation at pH 5 after addition of H_2O 1 : 1, according to ANDERSSON and co-workers (1985). This *Vicia faba* globulin (IV) contains about 50% 7 S globulin and 50% 11 S globulin. The preparation of gels was realized as described previously (SCHMANDKE et al., 1981a) under conditions of thermal denaturation at the maximum shear modulus: $T_D \approx 373$ K and

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$t_D \approx 60$ min for the non-acetylated *Vicia faba* protein and $T_D \approx 373$ K and $t_D \approx 40$ min for the acetylated one. The shear modulus of gels was determined with a TM-SM-L dynamometer (Instron) using the spherical indentor penetration method under stress relaxation (BIKBOV et al., 1979a, 1979b; LEE & RADOK, 1960) between 5 °C and 60 °C.

2. Results and discussion

Figures 1—4 show the stress relaxation curves of the 4 applied *Vicia faba* proteins at different temperatures. These curves originated from the recorder inside in the testing equipment. It is necessary to determine the value of the shift factor a_T for the calculation of the activation enthalpy ΔH by the Arrhenius relationship:

$$a_T = a_{T0} \exp\left(-\frac{\Delta H}{RT}\right), \text{ and}$$

$$\ln a_T = \text{const} - \frac{\Delta H}{R}\frac{1}{T}, \text{ resp.,}$$

where T is the temperature in degree Kelvin.

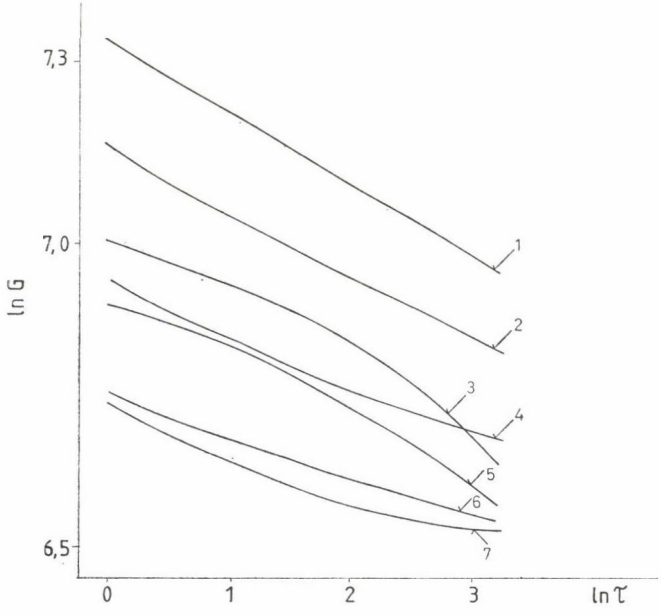

Fig. 1. Dependence of shear modulus G of *Vicia faba* protein gels (17%) on temperature t; degree of acetylation = 0%; pH = 7; τ = time. 1: 5 °C; 2: 10 °C; 3: 59 °C; 4: 20 °C; 5: 51 °C; 6: 40 °C; 7: 30 °C

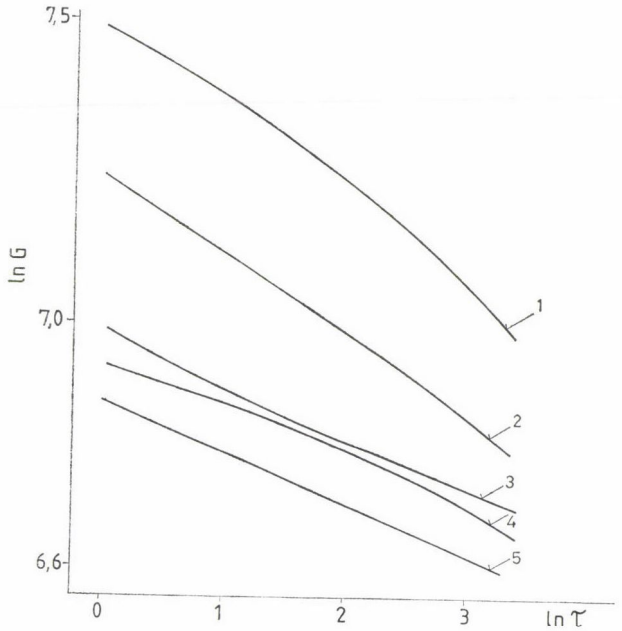

Fig. 2. Dependence of shear modulus G of *Vicia faba* protein gels (17%) on temperature t; degree of acetylation = 45%; pH = 7; τ = time. 1: 4 °C; 2: 11 °C; 3: 23 °C; 4: 44 °C; 5: 32 °C

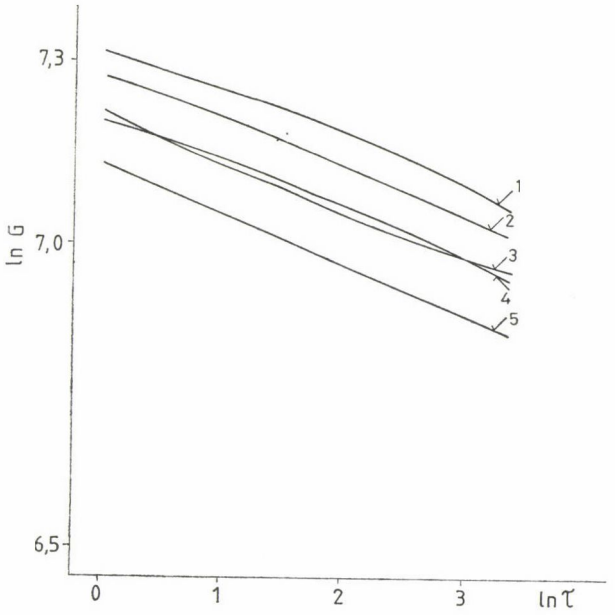

Fig. 3. Dependence of shear modulus G of *Vicia faba* protein gels (17%) on temperature t; degree of acetylation = 78%; pH = 7; τ = time. 1: 50 °C; 2: 11 °C; 3: 20 °C; 4: 40 °C; 5: 30 °C

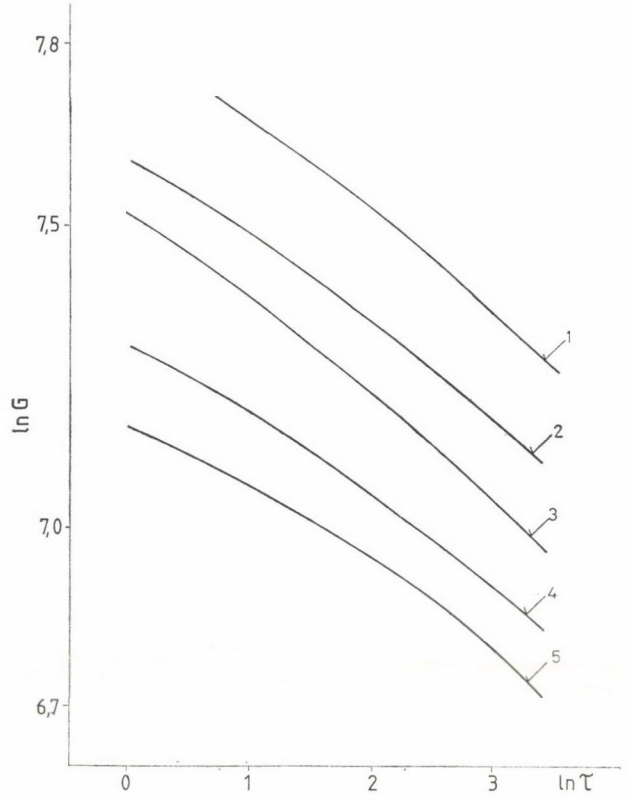

Fig. 4. Dependence of shear modulus G of *Vicia faba* protein gels (17%) on temperature t extracted at pH 8 with 0.37 M NaCl; degree of acetylation = 0%; pH = 7; τ = time. 1: 2.5 °C; 2: 11.5 °C; 3: 20 °C; 4: 30 °C; 5: 37 °C

In order to obtain the shift factors a_T the so-called time — temperature superposition principle or the principle of reduced variables were to be used. The relaxation shear modulus G is the stress relaxation divided by the strain (MATSUOKA & KWEI, 1979). The stress relaxation curves at different temperatures will be shifted towards a single temperature-invariant "master curve". It will be expected that the stress relaxation curves in dependence on rising temperature are deeper in the figures. That is an assumption for the construction of a master curve. This is valid for the stress relaxation curves of soy-bean globulin gels in a temperature range of 5 °C to 70 °C (BIKBOV et al., 1981).

In Figs. 1—3 it is shown that in this sense the temperature behaviour of the *Vicia faba* protein gels above temperature 30 °C, is different.

Therefore, the shifting of the stress relaxation curves towards a temperature-invariant master curve is only possible for a temperature up to about 30 °C. The data in Table 1 show the values of activation enthalpy for all

Table 1

Activation enthalpy of Vicia faba *protein gels*

Signs	Samples	ΔH (kL per mole)
I	*V.f.* protein gel degree of acetylation 0%	154
II	*V.f.* protein gel degree of acetylation 45%	114
III	*V.f.* protein gel degree of acetylation 78%	77.3
IV	*V.f.* protein gel NaCl extraction	84.8

investigated samples considering only the temperature range up to about 30 °C.

With this method it is not possible to determine the activation enthalpy for these *Vicia faba* protein gels above 30 °C. It is assumed that the structure of these gels is stable up to 30 °C and that their structure is perhaps modified with rising temperatures above 30 °C. The values of activation enthalpy depend on the acetylation degree. Higher values of ΔH mean higher values of relaxation time and therefore higher relative relaxation modulus G/G_0. As already discussed by BIKBOV and co-workers (1981) the activation enthalpy is connected with the so-called free volume, higher activation enthalpy means a lower free volume.

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THE RELATIVE IMPORTANCE OF SENSORY ATTRIBUTES FOR FOOD ACCEPTANCE

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This paper considers two measures of relative importance of attributes — self rated importance and measures of "annoyance" for statements about specific product defects. For liking attributes, panelists can easily rate the relative importance for each sense dimension (viz., good appearance vs. good taste, etc.). However, they cannot do so for sensory attributes, where importance loses its meaning (e.g. importance of sweetness, importance of darkness). On the other hand, when panelists respond to statements about specific product defects, by rating overall annoyance or irritation, the panelists can easily rate the importance of these defects, as they detract from overall acceptance. These annoyance ratings reflect the relative importance of the sensory attributes.

Keywords: sensory analysis, sensory attributes, marketing

Food researchers who work with either expert or consumer panelists know that these panelists can rate stimuli on a wide variety of attributes. However, at the same time, probing into the relative importance of these attributes reveals that some attributes play an important role, whereas others play a less important, and perhaps trivial role.

Descriptive sensory analysis with experts, using either the Flavor Profile Procedure (CAUL, 1957) or the Quantitative Descriptive Analysis Method (STONE et al., 1974) do not take into account the relative importance of the attributes. For descriptive analysis, with experts acting as the measuring instruments, the researcher has no other criterion by which to rank the order of the attributes in terms of importance. However, if the researcher can acquire consumer ratings of acceptance for the same product set, one can relate attribute levels to overall acceptance, and thus generate a rank order of importance.

Relatively little has appeared in the scientific literature pertaining to attribute importance. Marketing researchers acquire consumer ratings of attribute importance by direct rating of importance. In the main these research programs deal with attitudes about products, rather than with sensory characteristics of products. Researchers who study sensory characteristics often use correlation coefficients to measure the strength of a linear relation between sensory attribute level and overall liking (MOSKOWITZ, 1981). However, if the relation between liking and attribute level follows a parabola (e.g., an inverted

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U shaped function), then the straight line correlation analysis fails to measure importance. Depending upon the distribution of points on the parabola (relating to attribute level) the correlation coefficient may assume positive, zero, or negative values, even though only one relation (viz., the parabola) actually exists.

In a product evaluation study the investigator can instruct the panelists to rate the relative importance of specific characteristics. These instructions sound easy, and appear easy to administer, but not as straightforward to interpret. For instance, panelists can rank the relative importance of good appearance, good aroma, good flavour/taste, and good texture as they generate overall acceptance. Panelists easily perform this task. High values for the rated importance of flavour, and lower values for appearance, for instance, means that panelists want a good flavour, and feel this more important than good appearance.

For non-evaluative attributes, such as perceived flavour intensity or darkness of color, the approach just outlined above does not work. What exactly should the panelist do to rate the attitudinal importance of "flavour"? Does this mean the importance of "good flavour", or the importance of having the "right flavour" (whether type, or level)? Furthermore, do panelists know the relative importance of the "right level" of an attribute? Does over-delivering on the attribute generate equivalent loss in acceptance as does under-delivering? All of these questions remain unanswered because panelists have a difficult time even understanding the concept of "relative importance" when they deal with a non-evaluative, sensory attribute.

The "annoyance" generated by product defects

One way to develop measures of relative importance for non-evaluative sensory attributes consists of presenting the panelists with statements about specific product defects. The panelists can then rate an attribute such as "annoyance" or "loss of acceptance." Little or no literature has appeared using this technique. This paper explores the use of the technique for a variety of categories, attributes, and consumers.

1. Materials and methods

In all of the studies listed below panelists followed virtually the same format, as follows:

Test procedures

Step 1 — Panelists pre-recruited to participate by means of a telephone interview, qualified to participate, and then invited to participate for an extended (2–4 h) test session.

Step 2 — Panelists show up, in a group of 25 individuals, prepared to participate.
Step 3 — Attending interviewer leads panelist through a practice exercise, to insure comprehension.
Step 4 — Panelist rates products, in random order, profiling each product on a variety of attributes. After each product rating the panelist has his/her ratings checked for completeness and comprehension by an attending interviewer.
Step 5 — Panelist finishes products.
Step 6 — Panelist rates annoyance of product defects, or rates importance of product attributes.
Step 7 — Panelist paid and dismissed.

The key to the studies involves a pre-recruitment of panelists to participate for a test session lasting several hours. During this time the panelists evaluate a number of prototypes of a product, whether these represent quantitative variations of a limited set of ingredients, or qualitatively different products, such as varying flavours. Afterwards, the panelists rate the relative "annoyance" of specific product defects (e.g., the product tastes "too salty"). The panelist rates statements of defects presented in one of two formats:

— Four graded defects (much too much, slightly too much, slightly too little, much too little).
— Two graded defects (much too much, much too little)

In all cases the panelists use a 0—100 point annoyance scale (0 = no annoyance at all, 100 = extreme annoyance) for annoyance ratings. Furthermore, the same panelists use an anchored 0—100 point scale to rate sensory attribute levels and degree of liking. These scales and instructions are as follows:

Rating annoyance — instructions and scale

The manufacturer of these (example — coffees) products would like to know about product defects, and their importance to you. Suppose that you had purchased an instant coffee which tasted "too bitter" for your own personal taste. How annoyed would you feel? Use the scale listed below:

(0 = not annoyed, 100 = very annoyed)

Note that you can use the entire scale. Where would your feelings fall?

Now, I will present to you statements about product defects. For each defect, please rate how annoyed you would feel, using the scale listed above. You may use any number on the 100 point scale you wish, to show your feelings.

2. Results and discussion

2.1. Relative importance of sensory attributes by annoyance ratings. General trends

The basic analysis of relative importance consists of the average annoyance ratings assigned by panelists. Table 1 shows the results for bread, Table 2 shows the results for snack chips, and Table 3 shows the results for instant coffee.

Table 1

Annoyance for specific product defects.
Breads

	Much too little	Slightly too little	Just right	Slightly too much	Much too much	
Too light	46	30	0	44	65	Too dark
Too light weight	60	43	0	49	71	Too heavy weight
Too coarse texture	51	49	0	39	49	Too fine
Too weak aroma	57	48	0	52	70	Too strong aroma
Too weak flavour	71	49	0	58	73	Too strong flavour
Low sweetness	50	48	0	54	69	Too sweet
Not chewy enough	60	59	0	59	80	Too chewy

n = 45 panelists, consumers of white bread

Table 2

Annoyance for specific product defects.
Snack chips

	Much too little	Slightly too little	Just right	Slightly too much	Much too much	
Too light	25	22	0	42	74	Too dark
Too thin	31	25	0	42	57	Too thick
Too small	65	48	0	20	25	Too large
Too weak aroma	43	37	0	34	41	Too strong aroma
Too little saltiness	41	34	0	59	73	Too salty
Too weak flavour	61	48	0	24	33	Too strong flavour
Too dry	44	28	0	37	81	Too oily
Too soggy	89	83	0	12	13	Too crispy

n = 95, consumers of snack chips

Table 3

Annoyance for specific product defects.
Instant coffee

	Much too little	Slightly too little	Just right	Slightly too much	Much too much	
Too light granules	38	25	0	40	59	Too dark granules
Too fine granules	45	33	0	30	43	Too grainy granules
Too weak aroma	66	48	0	36	51	Too strong aroma
Not oily enough in cup	23	21	0	58	79	Too oily in cup
Too weak flavour	79	57	0	44	68	Too strong flavour
Too smooth	20	17	0	60	83	Too harsh
Too little bitterness	30	29	0	66	88	Too bitter
Too little aftertaste	43	34	0	55	78	Too strong aftertaste

n = 140, consumers of instant coffee

These initial results suggest the following trends:

- Attribute defects do generate different levels of annoyance, and therefore of importance.
- Quite often, flavour defects outweigh texture defects, which in turn outweigh appearance defects. However, the particular rank order depends upon the specific product.
- Defects in product sensory characteristics do not exhibit symmetry. For instance, "too salty" often generates more annoyance than "not

Table 4

Annoyance rating for product defects. Instant coffee by usage pattern

Caffeinated vs. non-caffeinated users
Heavy users (7 + cups per day)
vs. light (1–2 cups per day)

	Caffeine usage		Amount of coffee consumed	
	decaffeinated	caffeinated	heavy (7+)	light (1–2)
Base size (panelists)	60	80	45	95
Dry appearance in cup				
Too dark	38	38	32	47
Too light	59	58	54	60
Too fine	43	46	43	45
Too grainy	40	45	35	50
Black coffee prepared				
Aroma too weak	64	66	69	62
Aroma too strong	49	53	51	58
Not oily enough	22	23	19	24
Too oily	78	80	81	76
After whitener added				
Too dark	45	47	42	46
Too light	68	64	66	65
Flavour too weak	78	80	82	75
Flavour too strong	65	71	71	72
Too bitter	88	88	88	89
Not bitter enough	30	28	34	27
Too much aftertaste	76	80	79	81
Too little aftertaste	34	33	34	34
Too much caffeine	56	75	51	74
Too little caffeine	33	21	25	23
Too robust	43	40	41	42
Not robust enough	59	61	60	58

Note — 140 consumers (same total panel as rated annoyance value in Table 3).

salty enough." Or, "too soggy" generates substantially greater annoyance than "too crisp" (for a snack chip).

– Some defects generate little change in annoyance as they increase from "slight" to "very much" (example: coarseness of bread texture). Other defects generate a large increase in annoyance as they increase in magnitude (example: heaviness of bread).

2.2. Individual differences

Do individuals differ in the degree to which they respond to defects? To answer this question we looked at demographic breaks in the population (e.g., by age, by market, by product usage). Table 4 shows some of the annoyance ratings assigned to defects of instant coffee, by caffeinated vs. non-caffeinated users, and by heavy vs. light drinkers. To some extent, Table 6 suggests that there do exist modest, not strong differences among consumers. Table 5 shows a similar demographic breakout, this time for a multi-grain snack chip. Note again the similarity of annoyance ratings, across segments. However, for users of brand "A" (a greasier product) note less annoyance for too much greasiness than for users of brand "B" (a much drier product).

Individuals differ in acceptance of foods. Tables 4 and 5 presented individual differences as defined by demographic segments. However, one

Table 5

Annoyance for product defects in snack chips

	Users of brand A (greasier variety)	Users of brand B (dry, lighter variety)
Base size	30	25
Too dry appearance	47	43
Too oily appearance	80	82
Too small	63	67
Too large	32	21
Too thin	33	30
Too thick	58	56
Too light	29	23
Too dark	73	74
Very weak aroma	49	39
Very strong aroma	42	40
Too dry (feel in mouth)	32	23
Too oily (feel in mouth)	77	82
Not salty enough	41	41
Too salty	73	73
Too crispy	10	11
Too soggy	82	83

Table 6

Annoyance ratings for two sensory segments of snack cake

	Total	High sweet, low flavour seekers	Low sweet, high flavour seekers
Base size	80	50	30
Flavour too weak	48	47	49
Flavour too strong	65	68	60
Too sweet	66	64	69
Not sweet enough	56	60	50
Too thick	46	53	35
Too thin	63	69	54
Aroma too weak	42	37	50
Aroma too strong	61	68	50
Texture too grainy	61	61	60
Texture too smooth	27	28	26
Too dry	74	77	69
Too moist	45	44	46
Too spongy	43	42	44
Too firm	57	54	61

Table 7

Comparison of importance and ideal ratings. Margarine attributes

	Self rated[a] importance	Self designed[b] ideal margarine
Good colour	62	—
Good aroma	68	—
Good taste	88	—
Good texture	82	—
Deep colour	50	53
Light aroma	32	36
Sweetness	43	46
Saltiness	29	31
Buttery taste	63	69
Oiliness	10	12

[a] Panelist scaled relative importance.

[b] Panelist profiled the sensory characteristics of their ideal margarine, just as if it represented another margarine that they tested.

can also segment individuals on the basis of sensory preferences to products. Do panelists in these sensory preference segments exhibit different annoyance ratings for product defects (and thus different levels of importance for sensory attributes)?

To answer this question required segmenting panelists by sensory preferences. In a study of snack cakes varying in the level of sweetness and flavour,

it became feasible to segment the consumer into two distinct groups — a group of 50 who exhibited clear preferences for sweet, but low flavour cakes, and a group of 30 who exhibited clear preferences for less sweet, but high flavour cakes. Table 6 shows their ratings of annoyance for specific product defects. Note that these sensory preference segments exhibit clearly different annoyance ratings for the product defects (e.g., annoyance for an aroma that smells too strong, or a cake that does not taste sweet enough).

2.3. Direct ratings of importance of sensory attributes

This section deals with the direct rating of sensory attributes, for overall importance. The data for this section comes from the evaluation of a variety of stick margarine products. In the study the panelists first rated the sensory and acceptance characteristics of the products, using a 0–100 fixed point scale, and then rated relative importance. A zero rating meant "not important", whereas a 100 point rating meant "very important". The panelists also rated their self designed ideal stick margarine, using the same scale that they had used to rate the actual margarine products.

The profile of the ideal product, and the ratings of relative importance appear in Table 7. The panelists appear to have experienced difficulty in rating the importance of sensory attributes. The moderator in the test session had explained to the panelists that the importance rating for a sensory attribute pertained to the "importance of getting the product right on target for that attribute."

Table 7 reveals two things:

- The panelists could clearly rate importance of acceptance, for appearance, aroma, flavour and texture.
- However, when it came to ratings for specific sensory attributes, their importance ratings paralleled the self designed ideal. In effect, it appears that they had difficulty understanding the meaning of " importance" for these sensory characteristics. Quite possibly the concept of "importance that the product achieve the correct level of an attribute" has little or no meaning to the panelist. According to the importance ratings, "saltiness" has much less importance than sweetness and much more importance than "oiliness". Yet, for many categories we know that a product which tastes too salty generates a high rating of annoyance.

2.4. The hierarchy of attributes

The first general conclusion we can draw from this data pertains to the relative importance of difference sense characteristics. Consumers do show a hierarchy of importance, as measured by their annoyance ratings. Over-

delivery or under-delivery on some attributes has more of a deleterious effect than it does on others. However, until we ask consumers about annoyance, we do not know which sensory attributes play the critical role. For direct ratings of importance, consumers have a difficult time of rating relative importance.

2.5. Symmetry

Traditionally, researchers have looked for a single number to represent relative importance. This single number suffices when errors occur in one direction — either the product always delivers too little or too much vs. the ideal. For instance, for hedonics (liking), the ideal product always scores higher than the existing products, so all products under-deliver relative to the ideal. In such cases a single number suffices to indicate the importance of degree of underdelivery. On the other hand, for sensory attributes, a product can over-deliver or under-deliver. Furthermore, these two types of errors in the product do not generate the same annoyance, meaning that they possess different levels of importance. As noted previously, no hard nor fast rules apply here, to indicate which error (over- vs. under-delivery) generates more annoyance, and thus more importance. The degree of symmetry or asymmetry depends upon the product, and the particular attribute of that product.

2.6. Use of importance data

Traditionally, product testers and sensory analysts gather ratings from panelists using a large number of attributes. These attribute profiles guide product developers to reach a target product, or guide quality control in terms of deviations of a product from a "gold" standard.

If, in fact, sensory attributes do exhibit different levels of importance to acceptance, than the deviations between a prototype product and a control product possess different levels of criticality. Large deviations from a standard may signal serious problems, if the attribute on which the deviation occurs has a great deal of importance to the consumer. Conversely, the same magnitude of deviation may generate little or no problem at all, if the attribute in question has little or no importance.

3. Conclusions

— If we measure importance by ratings of „annoyance", the panelists show a hierarchy of importance of sensory attributes, as they influence acceptance.

— This hierarchy did not show up by self ratings of importance of attributes, at least in one experiment (viz. with margarine).

— Rating the degree of "annoyance" for stated product defects, on sensory attributes, may be used as a method of eliciting the relative importance value of the attribute. The panelist rates the degree of "annoyance" of deviations between the ideal product, and a described product, on the sensory attribute. High levels of annoyance correspond to high levels of importance.

— The criticality of such deviations from a desired attribute level does not show symmetry. Quite often "too much" of a characteristic produces more annoyance than too little of the characteristic.

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STUDY INTO THE EFFECT OF DIETS CONTAINING VARIOUS AMOUNTS OF SOYA FIBRE AND FAT IN RATS

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Wistar rats (grown at the National Institute of Food Hygiene and Nutrition, OÉTI, Budapest) were fed isoenergetical diets with 4 or 12% crude soya fibre content under conditions of adequate (5%) and excessive (20%) fat consumption for six weeks.

The high fibre diet caused a moderate decrease in the body mass mainly with adequate fat intake of the rats.

Serum total cholesterol and HDL cholesterol contents were unchanged with each diet but serum triglyceride level decreased after consumption of the diet with high soya fibre and sunflower oil content.

High soya fibre consumption resulted in a significant decrease of the elevated liver total lipid content induced by normal fibre but fat-rich diet. The iron, copper, zinc and lead contents of the liver were unchanged.

Keywords: Soya fibre, isoenergetical diet, lipid metabolism

A change in the fibre content of the diet is considered to be an important preventive measure not only in the treatment of various diseases of the digestive organs, but also for diseases associated with disturbances in the carbohydrate and fat metabolism. Since the efficiency of dietary fibre is determined by the quality and quantity of the fibre, it seemed reasonable to examine soya fibre in animal experiments, particularly as very few literary data are available on the subject (TSAI et al., 1980; LIENER, 1981).

1. Materials and methods

The aim of the experiments was to determine the effect of an increase in the fibre content of the diet on certain serum and liver lipid parameters in the case of normal, adequate and excessive fat consumption. Groups of 10 adult male OÉTI Wistar rats were fed for 6 weeks with the semisynthetic feeds listed in Table 1.

In order to vary the soya fibre content, heat-treated ground soya hull prepared and analysed for experimental purposes at the Vegetable Oil Research

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Table 1

Semisynthetic diets based on soya

Nutrients and crude fibre content	Diets[a]			
	A	B	C	D
	(%)			
Protein content (50% of animal origin)	18	18	18	18
Fats	5	20	5	20
Crude fibre	4	4	12	12
Carbohydrate	73	58	65	50
Total	100	100	100	100

[a] Diets consist of
Soya meal (heat-treated, extracted)
Ground soya hulls (heat-treated)
Sunflower oil (soya oil under 1%)
Starch
Casein
Salt and vitamin mixture (2%)

Institute (Budapest) was mixed with extracted, heat-treated soya meal in various proportions. The analytical data of the ground soya hull were as follows: moisture content 5.0%, oil content 5.1%, protein content 16.4%, crude fibre content 28.3%, trypsin inhibitor content 5.8 TIU per mg. (The composition was analysed using the procedures specified in HUNGARIAN STANDARDS, 1976, 1977 and 1981.)

According to the literature data (SANELLA & WHISTLER, 1962) the hull contains α-cellulose (50%), lignin (8%), hemicellulose A (10%), hemicellulose B (6%), D-oxylose, L-arabinose, D-glucose and galactomannan. The analyses made by other authors (ASPINALL & BOGHIE, 1967) indicate that they consist mostly of cellulose (40%), hemicellulose (10%), polysaccharides (10–12%) and galactomannan (9–11%).

The soya meal, analysed in accordance with the HUNGARIAN STANDARDS (1976, 1977 and 1981), was found to contain 38% protein, 2.2% oil and 6% fibre, while the trypsin inhibitor content was 4 TIU per mg.

The protein content of the diets was complemented with casein in such a way that half of the total protein content consisted of soya protein and the other half of casein. The fat content was adjusted by the addition of sunflower oil. Nutrients and crude fibre contents of the diets were as follows: diet A contained 4% crude fibre and 5% fats and diet B 4% crude fibre and 20% fats. The crude fibre content of diets C and D was raised to 12%, while the fat content was 5% and 20%, respectively. Rats were fed isoenergetically.

The following tests were carried out in the serum and liver of rats fed for six weeks:

— mass of fatty tissue, determined by mass measurement;
— fat content of the liver extracted according to FOLCH and co-workers (1957) and analysed according to ZÖLLNER and KRISCH (1962);
— cholesterol content of the serum and liver analysed using the Bio-La test;
— HDL-cholesterol content of the serum, precipitated according to BURSTEIN and co-workers (1970) and analysed using the Bio-La test;
— copper, zinc, iron and lead contents of the liver were analysed according to GERGELY and LINDNER-SZOTYORI (1976).

Analytical results in rats, fed with a natural rat diet (4% crude fibre, 5% fat) were used as "normal" values. Significances were calculated by Student's t test.

2. Results and conclusions

Consumption data show that the energy uptake was generally similar, but the animals ate somewhat more of diet C than of the others (Table 2).

Table 2

Energy intake day per 100 g body mass of rats

	Diets			
	A	B	C	D
kJ	83.5	87.3	93.2	89.7

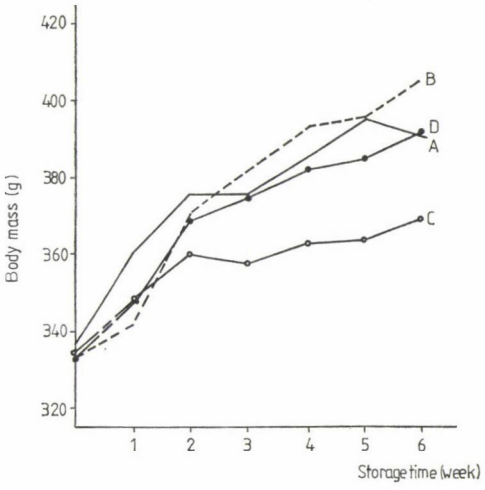

Fig. 1. Trend in body mass.
A: 5% fat } 4% fibre
B: 20% fat }
C: 5% fat } 12% fibre
D: 20% fat }

The body weight gain, however, was the lowest for group C (Figs 1, 2). This may be caused by a reduced nutrient absorption (especially fat) as consequence of the fibre induced increased activity of the gastrointestinal tract. It is possible that the effect of certain residual antinutritive substances in the heat-treated soya manifests itself, too, causing a reduction in the utilization of nutrients.

As an index of the gain in adipose tissue, the weight of the epididymal fat tissue was measured. As can be seen, in the case of excessive fat intake,

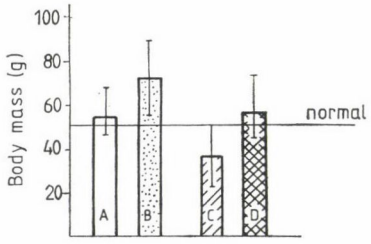

Fig. 2. Change in body mass in 6 weeks. For diets A—C see legend in Fig. 1

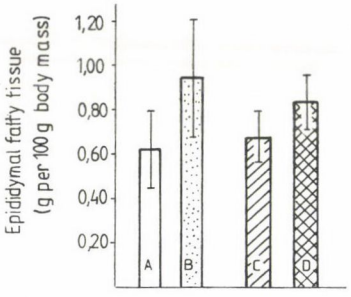

Fig. 3. Quantity of epididymal fatty tissue per 100 g body mass A–B: $P < 0.01$; C–D: $P < 0.01$

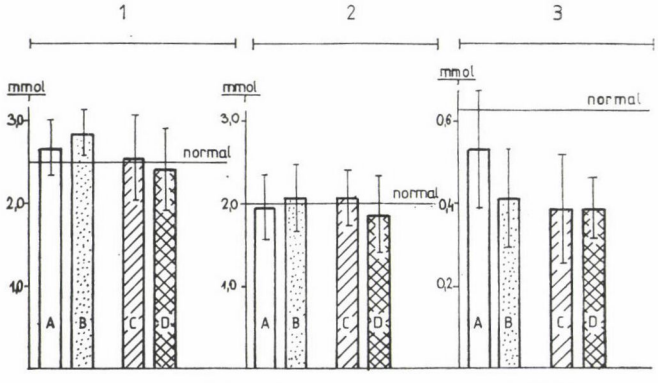

Fig. 4. Levels of serum lipids. 1: total cholesterol; 2: HDL-chlolesterol; 3: triglycerides. A–D: $P < 0.05$

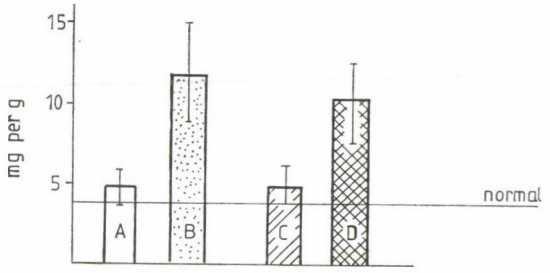

Fig. 5. Total cholesterol content of the liver. A–B: $P < 0.001$; C–D: $P < 0.01$; A–C: Ø; B–D: Ø

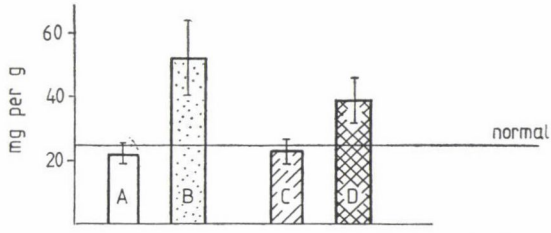

Fig. 6. Triglyceride content of the liver. A–B: $P < 0.001$; C–D: $P < 0.01$; A–C: Ø; B–D: Ø

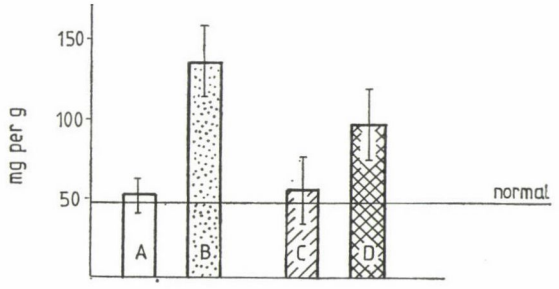

Fig. 7. Total lipid content of the liver. A–B: $P < 0.001$; C–D: $P < 0.01$; A–C: Ø; B–D: $P < 0.05$

a high consumption of fibre led to a reduction in the quantity of the epididymal fat tissues (Fig. 3). The relative weights of the organs (g per 100 g b.w.) did not change for any of the diets.

Among the serum lipid parameters, the consumption of a diet containing 12% fibre had again a moderating effect on the triglyceride level when there was excessive fat intake (Diet D). Diets containing a combination of soya fibre and sunflower oil (which is known to reduce serum lipids), resulted in a lower triglyceride level in all groups compared to rats given normal rat diet (Fig. 4). Total cholesterol and HDL cholesterol of the serum were unchanged.

With higher fibre intake in relation to the total cholesterol level of the liver, no significant change was observed. Under the effect of excessive fat

Table 3

Metal content (μg per g wet tissue) of rat organs at various consumption levels of fibre (percentage of crude fibre content) and fat

	Copper		Iron		Zinc		Lead	
Fibrecontent	4%	12%	4%	12%	4%	12%	4%	12%
Fat content 5%								
Liver	3.2 ± 1.3	3.3 ± 0.9	110 ± 19	119 ± 21	28.8 ± 4.4	34.3 ± 7.2	0.52 ± 0.3	0.7 ± 0.3
Kidneys	6.1 ± 1.4	5.2 ± 1.9	79 ± 10	84 ± 10	21.0 ± 9.8	18.8 ± 3.8	1.5 ± 0.9	1.5 ± 1.0
Heart	4.0 ± 1.0	4.4 ± 0.8	104 ± 34	100 ± 31	15.2 ± 3.3	20.8 ± 1.4	0.63 ± 0.4	0.89 ± 1.0
Fat content 20%								
Liver	3.6 ± 0.9	3.2 ± 0.9	127 ± 37	107 ± 34	30.0 ± 6.0	29.2 ± 5.0	0.69 ± 0.4	0.62 ± 0.2
Kidneys	4.6 ± 1.2	5.2 ± 1.4	99 ± 32	92 ± 33	18.5 ± 6.4	20.0 ± 3.7	2.0 ± 1.0	2.9 ± 1.0
Heart	4.9 ± 2.8	5.1 ± 1.4	125 ± 83	93 ± 11	18.3 ± 6.5	19.0 ± 6.4	0.69 ± 0.4	0.78 ± 0.7

intake the total cholesterol level of the liver rose in animal groups fed both diets (Fig. 5).

As regards the triglyceride content of the liver in connection with high fibre diet consumption, again no significant difference was found, but the increase in triglycerides caused by excessive fat intake was moderate in tendency when the diet contained 12% soya fibre (Fig. 6).

The total lipid content of the liver showed a similar trend, however, the increase in total lipid content of the liver associated with an excessive intake of fat was significantly reduced by a diet containing 12% soya fibre (Fig. 7).

The trend in the iron, copper, zinc and lead contents of the various organs (liver, kidneys, heart) of the animals was also examined. In this respect, no significant changes were observed as the result of an increase in fibre consumption (Table 3).

According to our results, a diet containing a high amount (12%) of soya crude fibre and sunflower oil reduced the serum triglyceride level compared to the normal control levels but serum cholesterol and HDL-cholesterol levels were unchanged. An increase in the soya fibre of the diet moderated the negative effects of excessive fat consumption (greater body mass increase and total lipid accumulation in the liver). Under the given experimental conditions no significant change was found in the iron, copper, zinc and lead contents of the various organs.

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GENOTOXICITY TEST OF IRRADIATED SPICE MIXTURE BY DOMINANT LETHAL TEST

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Dominant lethal test was performed in Sprague Dawley male rats prefed 25% irradiated spice mixture in a semisynthetic diet for 3 months from weaning.

The spice mixture used in meat industry was composed of 55% non-pungent ground paprika, 14% black pepper, 9% allspice, 9% coriander, 7% marjoram, 4% cumin, 2% nutmeg.

Microbial count of the spice mixture was reduced with 15 kGy from a ^{60}Co source.

As controls 10 male rats in each group received the semisynthetic diet spice free or containing untreated spice mixture. 150 mg per kg body weight cyclophosphamide was administered i.p. to males in the positive control group 48 hours before mating. Two virgin females per male were replaced weekly during 4 successive weeks.

Among the groups there were statistically significant differences only in relation to the positive control group in the DLT parameters investigated.

According to the results of DLT neither untreated nor irradiated spice mixture are germ cell mutagens.

Keywords: dominant lethal test, genotoxicity test, irradiated spices

Spices with low microbial content are required by the food industry worldwide (GOTTSCHALK, 1977; GERHARDT & LADD EFFIO, 1982; FARKAS, 1982).

Irradiation is a modern, suitable method for sterilization or for reduction of the microbial count in spices. However, before practical application of this process the wholesomeness of the treated product should be and has been examined in animal feeding studies (BARNA, 1973a, 1976, 1977, 1980, 1981).

Recently one of the most important factors in the estimation of health risk is the possible genotoxic effect of any food treatment or additive substances, etc.

Relatively few mutagenicity tests (BARNA, 1979; ANON, 1980) were performed on irradiated spice mixture (Table 1) or individual spices, except onion.

Among them on irradiated paprika and its extracts host mediated assay and Salmonella/microsome tests were carried out (CENTRAL FOOD RESEARCH INSTITUTE, 1977). Urine, blood and spleen cell suspension from rats and mice fed paprika were used in Ames tests (FARKAS et al., 1981), in prophage induction test (FARKAS & ANDRÁSSY, 1981), in DNA synthesis and repair tests (KOVÁCS & EMŐD, 1975), respectively. Drosophila sex linked recessive lethal

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and mosaic tests were also involved in the genotoxic investigation of paprika (SZABAD, 1980). In mammalian test systems chromosome analyses (BARNA, 1973a) and micronucleus test (CHAUBEY et al., 1979) were performed.

On irradiated black pepper Ames tests were carried out in urine of rats (FARKAS et al., 1981). Prophage induction tests were used on blood of rats

Table 1

Genotoxicity tests of irradiated spice mixture

Spices	Dose (kGy)	Tests	Effects	
			mutagenic	non-mutagenic
Spice mixture (same as in this DLT) extract	0, 15, 45	Salmonella/microsome TA 1537, 1538, 98	—	FARKAS et al., 1981
Urine of rats fed 25% spice mix. for 6 days	0, 5, 15	Salmonella typhimurium TA 1535, 1537, 1538, 98, 100	—	FARKAS et al., 1981
Blood of rats fed 25% spice mix. for 6 days	0, 5, 15	Lysogen E. coli K-12 prophage induction	—	FARKAS & ANDRÁSSY, 1981

kept on diet containing black pepper (FARKAS & ANDRÁSSY, 1981). Tests cited have shown no mutagenic activity of irradiated spices or spice mixtures. However mammalian mutagenicity tests are so far lacking for mixed spices.

The purpose of the present investigation was to supplement the toxicohygienic feeding studies (BARNA, 1976, 1977) by the dominant lethal test on rats fed spices — used worldwide in the food industry — untreated or radappertized.

1. Materials and methods

1.1. Experimental animals and their housing

Sprague-Dawley CFY rats from National Laboratory Animal Breeding Institute (Gödöllő) were used in the DLT. Weaned males were 3 weeks old at the beginning of the feeding period. Number of males was 10 per group. Nulliparous females of the stock colony were 8–10 weeks old. Males were housed 5 per wire cage. During the mating period each male was caged separately with 2 virgin females.

Animals were identified by ear and footfinger number-system.

The animals were housed in an environment at 20–23 °C, 60–70% humidity and 10h artificial light, 14h dark cycle. Air changes were ensured by automated ventilation program.

1.2. Groups, diets and feeding regime

Animals received feed and water ad libitum. A special self-feeder was used to avoid spoilage (BARNA, 1973b).

The formulation of the diets and their chemical composition are shown in Table 2.

The spice mixture contained 55% non-pungent paprika (from Canned Food and Paprika Industrial Comp., Kalocsa), 14% black pepper, 9% allspice, 9% coriander, 7% marjoram, 4% cumin, 2% nutmeg. This mixture represents a composition generally used in the meat industry.

Because of the coarse meal character of the diets, wood stick for nibbling was given to ensure normal tooth wearing.

Four groups were used in the experiment. Two control groups (S, S^+) received spice free semisynthetic diet. A third control group (SF) was fed a semisynthetic diet containing 25% spice mixture untreated; the experimental group (SFX) was kept on the same semisynthetic diet as males in SF group, but the spice mixture was irradiated.

A positive control group (S^+) received 150 mg per kg body weight of cyclophosphamide (VEB Jenapharm., DDR) in saline sol. i.p. 48h prior to mating. The use of CPA as a known mutagen (HEDDLE, 1979) is accepted in DLT (e.g. KNUDSEN et al., 1977; ROBERTS & RAND, 1978; SYKORA et al., 1979).

During the mating period males and females were kept on standard stock diet of the National Laboratory-Animal Breeding Institute.

Table 2

Composition of the diets

Components	Semisynthetic diet	
	spice free (%)	containing untreated or irradiated spice mixture (%)
Casein	10.00	15.00
Milk powder	20.00	35.00
Starch	20.00	10.50
Flour	20.50	10.00
Bran	0.74	0.74
Vitamin premix (Phylaxia)	0.23	0.23
Mineral premix (Phylaxia)	3.03	3.03
NaCl	0.50	0.50
Alfalfa roughage	25.00	—
Spice mixture	—	25.00
Chemical analysis:		
Dry matter	91.30	91.80
Crude protein	23.10	27.00
Crude fat	4.65	11.00
Crude fiber	6.88	5.73
N free extract	50.13	41.22
Ash	6.27	6.35

1.3. Irradiation of the spice mixture

It was carried out with the "Gamma cell" ^{60}Co source of the Central Food Research Institute, Microbiology Dept. Dosis of the radiation treatment was 15 kGy. Intensity of source was 0.5 kGy h^{-1}.

Feeding of the irradiated spice mixture started on the 5–9th day after irradiation.

1.4. Dominant lethal test (DLT)

DLT was performed according to EPSTEIN and ROHRBORN (1971).

At the end of the prefeeding period — which covered the whole spermatogenic cycle — each male was caged with 2 virgin females. Females were replaced weekly during 4 successive weeks. Every morning each female was examined for the presence of a vaginal plug. On the 13th day post coitum females were killed and scored for number of corpora lutea, counts of total implantation, live and dead (early and late death) embryos. Females not mated were also killed on the 13th day after the last day of mating period for detecting unobserved pregnancy.

In the detection of the corpora lutea only great yellow bodies were counted, uncertain small ones not (ERIKSEN & EMBORG, 1972).

Frequency of dominant lethality could be expressed and compared between the test and control groups by different data and indices. Since there is no undoubtful and generally accepted method of comparison, the most frequently used formulas were calculated as follows:

$$\text{Mating index} = \frac{\text{No. of dam pregnant}}{\text{No. of females mated}} \times 100$$

$$\text{Preimplantation loss (\%)} = \frac{\text{No. of corpora lutea} - \text{No. of total implants}}{\text{No. of corpora lutea}} \times 100$$

$$\text{Postimplantation loss (\%)} = \frac{\text{No. of total implants} - \text{No. of live implants}}{\text{No. of total implants}} \times 100$$

$$\text{Dominant lethality DL(\%)} = \left[1 - \frac{\dfrac{\text{No. of live embryos}}{\text{No. of corpora lutea}}\ \text{in test group}}{\dfrac{\text{No. of live embryos}}{\text{No. of corpora lutea}}\ \text{in control}} \right] \times 100$$

$$\text{Lethality factor } F_L(\%) = \left[1 - \frac{\dfrac{\text{No. of live embryos}}{\text{No. of pregnant dams}}\ \text{in test group}}{\dfrac{\text{No. of live embryos}}{\text{No. of pregnant dams}}\ \text{in control}} \right] \times 100$$

Table 3

Data on reproduction

Diet	Week		Mating index %	Corpore lutea per dam		Implantations per dam						Loss in preimplants per dam
				$\bar{x}$	±s	total	±s	live	±s	dead	±s	
S	1		90	16.67	3.412	14.39	2.500	13.56	2.549	0.83	1.150	2.28
	2		60	16.25	3.333	14.00	2.828	13.17	2.757	0.83	0.937	2.25
	3		75	15.47	2.065	13.80	1.207	13.47	1.302	0.33	0.617	1.67
	4		70	16.21	2.636	13.43	2.408	12.43	2.927	1.00	1.240	2.79
		$\bar{x}$	73.75	16.17	2.883	13.93	2.265	13.19	2.438	0.75	1.027	2.24
S^+	1		65	14.92	2.178	10.38	4.312	3.46	4.807	6.92	3.427	4.54
	2		85	15.76	3.751	10.47	3.590	4.00	3.640	6.47	2.741	5.29
	3		85	13.94	1.560	10.24	3.784	3.06	3.230	7.18	3.046	3.71
	4		85	15.35	2.060	12.41	2.980	5.06	3.381	7.35	3.481	2.94
		$\bar{x}$	80.00	15.00	2.582	10.91**	3.681**	3.92	3.730***	6.98	3.114***	4.09*
SF	1		85	15.47	2.477	13.71	2.365	12.65	2.448	1.06	0.899	1.76
	2		75	16.47	3.226	14.60	2.097	13.93	2.016	0.67	1.046	1.87
	3		75	15.93	3.104	13.00	1.647	12.60	1.549	0.40	0.632	2.93
	4		90	17.61	3.051	15.06	1.626	14.00	1.571	1.06	1.259	2.56
		$\bar{x}$	81.25	16.40	3.014	14.12	2.072	13.31	2.007	0.82	1.013	2.28
SFX	1		75	15.75	1.791	14.07	1.831	13.13	2.263	1.07	1.099	1.67
	2		100	15.65	2.300	13.85	3.281	13.20	3.381	0.65	0.587	1.80
	3		90	15.44	2.035	13.56	2.035	12.72	2.321	0.83	0.985	1.89
	4		95	16.26	2.535	13.32	3.559	12.74	3.493	0.47	0.772	2.95
		$\bar{x}$	90.00	15.78	2.183	13.68	2.797	12.94	2.916	0.74	0.872	2.10

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

$$\text{Mutation rate (\%)} = 1 - \frac{\dfrac{\text{No. of live embryos}}{\text{No. of total implants}}\ \text{in test group}}{\dfrac{\text{No. of live embryos}}{\text{No. of total implants}}\ \text{in control}} \times 100$$

1.5. Statistical analyses

Statistical analyses were made by the Computer Unit of the Central Food Research Institute, Budapest.

Results were analysed statistically by two tailed variance, Student's test and chi² test using computerised programs. For data of early death Freeman-Tukey transformation was applied.

2. Results

Mating index and reproduction biology findings scored at female autopsy (corpora lutea, live and dead implants) are summarized in Table 3.

There were no significant differences in any of the four weekly matings in fertility of males within the four groups. Mean pregnancy rates among the

Table 4

Frequency of dead implants per dam

Diet	Week	No. of dams	Dead implants per dam							
			0		≥1		≥2		≥3	
			♀	%	♀	%	♀	%	♀	%
S	1	18	10	56	8	44	5	28	1	6
	2	12	5	42	7	58	2	17	1	8
	3	15	11	73	4	27	1	7	0	0
	4	14	6	43	8	57	3	21	2	14
	$\bar{x}$	15	8.00	54	6.75	47	2.75	18	1.00	7
S+	1	13	0	0	13	100	12	92	11	85
	2	17	0	0	17	100	15	88	15	88
	3	17	0	0	17	100	16	94	16	94
	4	17	0	0	17	100	17	100	15	88
	$\bar{x}$	16	0***	0	16.00***	100	15.00***	93.50	14.25***	88
SF	1	17	6	35	11	65	7	41	0	0
	2	15	10	67	5	33	4	27	1	7
	3	15	10	67	5	33	1	7	0	0
	4	18	9	50	9	50	7	39	2	11
	$\bar{x}$	16	8.75	55	7.50	45	4.75	29	0.75	5
SFX	1	15	6	40	9	60	4	27	1	7
	2	20	8	40	12	60	1	5	0	0
	3	18	8	44	10	56	3	17	2	11
	4	19	13	68	6	32	3	16	0	0
	$\bar{x}$	18	8.75	48	9.25	52	2.75	16	0.75	5

*** $P < 0.001$

Table 5

Indices of dominant lethality

Diet	Week	Loss in preimplants (%)	F_L (%)		DL (%)		Loss in post-implants (%)	Mutation rate (%)	
			S	SF	S	SF		S	SF
S	1	13.68	—	—	—	—	5.77	—	—
	2	13.85	—	—	—	—	5.93	—	—
	3	10.80	—	—	—	—	2.39	—	—
	4	17.15	—	—	—	—	7.35	—	—
	$\bar{x}$	13.87	—	—	—	—	5.49	—	—
	$\pm s$	2.596	—	—	—	—	2.135	—	—
S^+	1	30.41	76.3	—	28.5	—	66.67	64.1	—
	2	33.58	65.8	—	64.4	—	61.80	57.8	—
	3	26.58	76.5	—	72.4	—	70.11	68.7	—
	4	23.69	64.0	—	58.1	—	59.24	54.8	—
	$\bar{x}$	28.56**	70.6	—	55 8	—	64.45***	61.3	—
	$\pm s$	4.331	—	—	—	—	4.869	—	—
SF	1	11.38	7.0	—	—0.5	—	7.73	2.0	—
	2	11.35	—6.0	—	—4.3	—	4.59	—1.0	—
	3	18.39	6.0	—	9.0	—	3.08	0.7	—
	4	14.48	—13.0	—	—3.7	—	7.04	0.4	—
	x	13.90	—1.0	—	0.4	—	5.61	0.4	—
	$\pm s$	3.334	—	—	—	—	2.159	—	—
SFX	1	10.67	3.0	—4.0	—2.5	—1.4	6.68	1.0	—1.0
	2	11.50	0.0	5.0	—4.1	0.2	4.69	—1.0	0.0
	3	12.18	5.6	—0.9	5.4	—4.0	6.19	3.9	3.0
	4	18.08	—2.0	9.0	—2.2	1.4	4.35	—3.0	—3.0
	$\bar{x}$	13.11	2.0	3.0	—3.3	—1.0	5.48	0.1	—0.3
	$\pm s$	3.372	—	—	—	—	1.132	—	—

** $P < 0.01$; *** $P < 0.001$

groups for the whole test period did not differ statistically, either. The proportion of females becoming pregnant was normal, corresponding to the rate observed in the stock colony.

No significant differences were detected in the number of corpora lutea among groups. However, in the positive control group implantation index ($P < 0.01$), number of live and dead embryos ($P < 0.001$), pre- and postimplantation loss ($P < 0.01$; $P < 0.001$, resp.) differ significantly from the others. No significant differences or excess figure over physiological limits or data for stock colony could be found in data among the successive weeks within the groups or in means among groups other then S^+.

Frequency distribution of dams with dead implants is shown in Table 4.

There was no difference among groups except S^+. In this positive control group there was no dam without dead implants. Here the rate of dams with ≥ 1, ≥ 2 or ≥ 3 dead implants were significantly higher ($P < 0.001$) than in any of the other groups.

Among the calculated indices (Table 5) postimplantation loss and mutation rate are direct parameters for expressing induced lethality. Dams in both

of the spice diet groups are on the same level in postimplantation loss as the control group. Mutation rate indices induced lethality only in the S^+ group. Inter-group data among weeks differ from each other within physiological variance. S^+ group shows again a significant ($P < 0.01$) difference from the other groups.

Preimplantation loss and other indirect indices such as dominant lethality %, lethality factor % show significant differences only in relation of S^+ to other groups. Within the spice groups and control groups there is no statistically significant difference among the data of weeks.

3. Discussion

Induced mutagenic effect could be estimated from direct or indirect indices in DLT. As a direct parameter an increase in postimplantation loss (which expresses the early embryonic death) is considered to be the most relevant sign in the assessment of dominant lethality (BATEMAN & EPSTEIN, 1971). Frequency of dead implants per dam is also a suitable indicator for estimating induced dominant lethality.

All the direct indices proved the irradiated spice mixture not to be mutagenic for germ cells.

Indirect calculations such as DL %, F_L % and preimplantation loss % involved into the investigation contain factors which might express maternal effects, too, beside genetic causes. In evaluation of new products, additive substances, food industrial treatments, etc. a wider approach is more preferable in the estimation of health risk. For this reason indirect mutagenic indices were calculated and evaluated in this DLT. All of them have also shown the mutagenic ineffectiveness of irradiated spice mixture.

In previous investigations it was found that feeding of the spice mixture regardless of irradiation before mating and during gestation caused some disturbances in reproduction. A more detailed research cleared up that black pepper fed at a high level (3.5% of the diet) is responsible for this effect (BARNA, 1976, 1980). Comparing data of this DLT to earlier findings it can be supposed that the reducing effect of unirradiated black pepper on the number of offsprings is rather a consequence of gonado- and embryotoxicity than a genotoxic phenomenon.

The mutagenicity testings performed up to now have shown no mutagenic effect of irradiated spices or spice mixture in the dose range practically used by the food industry. Results of a mammalian in vivo system obtained in the DLT are in accordance with the general conclusion that irradiated spices are not genotoxic.

We can also conclude from this DLT that the native spice mixture used causes no genotoxicity, either.

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COMPARATIVE EVALUATION OF UNTREATED AND RADURIZED CHILEAN AVOCADOES SHIPPED TO THE NETHERLANDS

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Avocadoes of Fuerte variety were picked mature green, treated with a hot water dip (10 min at 46 °C) then individually wrapped in PVC foil, irradiated with a gamma radiation dose of 25 Gy and shipped by boat at 7 °C from Chile to the Netherlands for investigation of keeping quality. Similarly packed untreated fruits served as controls. After receiving them in Holland, halfs of the batches were stored at 20 °C for 8 days while the other halfs of the batches were kept first for 8 days at 12 °C then moved to 20 °C for additional 10 days. The combination treatment and wrapping in PVC foil delayed and reduced the decay, and resulted in a better consumer quality (less discolouration, better appearance, flesh consistency and flavour). This small-scale trial shipment holds promise that a sea-transport of combined treated avocadoes from Chile to Western Europe would be feasible.

Keywords: avocadoes, combination treatment, radurization, trial shipment

In Chile, avocadoes are being grown in increasing quantities. Due to this, a high production of this fruit can be expected in the coming years. The opening of new markets to be reached by sea is therefore quite essential. However, sea transport takes a longer time and gives problems in maintaining a good quality of the fruits. Since avocadoes are consumed principally as fresh fruit, it is required by the importing countries that the fruits are of excellent quality.

Several studies have been conducted for shelf-life extension of avocadoes with gamma ray doses ranging from 0.1 to 2 kGy (Bramlage & Couey, 1965; Akamine & Wong, 1966; Brewbaker & Ross, 1966; Kahan et al., 1968; Kamali et al., 1972). These studies revealed, however, that such doses caused

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skin damage, and discolouration of the flesh of the fruit. In the Fuerte avocado approximately 0.1 kGy is the maximum dose this variety can tolerate and apparently ripening is delayed by this dose (AKAMINE & MOY, 1983). Combination treatment may help, however, to minimize irradiation dose and to avoid unwanted side effects of higher doses. THOMAS and BRODRICK (1977) have reported at first that doses of 10–30 Gy, accompanied by mild heat treatment were found to give a measure of delayed ripening with a minimum of phytotoxicity. Neither the irradiation-only treatment nor hot-water-only treatment was as effective as their combination. Subsequent studies conducted in Chile showed that Fuerte avocadoes given a mild heat treatment (46 °C for 10 minutes) plus very low dose (25 Gy) gamma irradiation, and wrapped with PVC foil were well preserved for 45 days of refrigerated storage (7 °C) followed by six days at room temperature (ANON., 1982, 1983; KARMELIC et al., 1983). Because of the promising results obtained, a sea-shipment of irradiated avocadoes from Chile to the International Facility for Food Irradiation Technology (IFFIT), Wageningen, The Netherlands, was carried out for comparative evaluation of untreated and radurized (combined treated) avocadoes.

1. Materials and methods

1.1. Test materials

Avocadoes of Fuerte variety were picked the last week of June 1983 by hand in a farm, 120 km north of Santiago de Chile, sorted to eliminate any damaged fruits, packed in cardboard boxes and sent in an unrefrigerated truck to the Nuclear Centre "La Reina", Santiago.

1.2. Methods

1.2.1. Treatments and packaging. Treatments and packaging were carried out at the Nuclear Centre. One day after harvest, the fruits were dipped in a water bath and heated for 10 minutes at 46 °C under gentle agitation. After this mild heat treatment the fruits were dripped, individually wrapped in PVC foil, and irradiated with a gamma radiation dose of 25 Gy. The dose rate was 5 Gy per minute. The irradiation was performed within 20–120 minutes after the hot water dip.

The PVC-wrapped fruits were packed for shipment in three cardboard boxes with hay at the bottom to serve as protection. Each box contained 13–14 pieces of fruits or 4–4.5 kg. Equal amounts of untreated fruits with the same packaging (in PVC wrapping and cardboard box) served as control. After treatments and packaging the fruits were kept at 7 ± 1 °C and 90% relative humidity until shipping.

1.2.2. Shipping transport. The experimental batches were transported to Valparaiso on 27 June 1983, where they were dispatched for Rotterdam in a refrigerated cabinet of the ship "Schwabenstein" of the Ultramar Agencia Maritima Ltda., while on voyage, the fruits were still kept at 7 °C. The shipment arrived in Rotterdam, the Netherlands, after one months, on 29 July 1983, and it was transported by car to the Research Institute ITAL, Wageningen, the Host Institute of IFFIT.

1.2.3. Post shipment storage. At ITAL, the shipment was temporarily stored for two days at 12 °C before unpacking. On 1 August 1983 both the untreated and the radurized (combined treated) batches were divided in two lots. Half of the batches were stored at 12 °C and 80–90% r.h. for 8 days, and then moved to 20 °C and 90–95% r.h. while the other half of the batches were stored all the time at 20 °C and 90–95% r.h. to allow ripening and too simulate conditions of retail distribution.

1.2.4. Quality evaluation. While in storage, the following investigations were carried out periodically. Each fruit was individually inspected and rated. For the inspection the fruits were unwrapped. From each lot six fruits were kept undisturbed until the end of post-shipment storage to estimate the effect of inspection.

1.2.4.1. Disease. — Each fruit was carefully inspected for disease, and the number of diseased fruits was expressed as percentage of total number of fruits in the lot.

1.2.4.2. Skin-colour. — Method of assessment for colour by visual rating was as follows:

Rating	*Description*
5	100% of skin surface is green
4	75% of skin surface is green
3	50 : 50%, green : brown
2	75% of skin surface is brown
1	100% of skin surface is brown

1.2.4.3. Softness measurement. — A non-destructive method was used. The fruit was placed in the dish of a modified Berkel balance (see Fig. 1) and the weight of the fruit was balanced until the pointer was within the scale. The screw was then adjusted until it touched the fruit and gave a difference of 0.5 g on the scale. The initial reading was noted and then a known weight of 300 g was placed in the other dish of the balance, and the deflection on the scale was recorded after 15 seconds. The difference of the final and initial reading is an indication of the softness of the fruit.

1.2.4.4. Weight-loss. — Each fruit was individually weighed. The difference between the initial weight at the beginning of the post-shipment storage

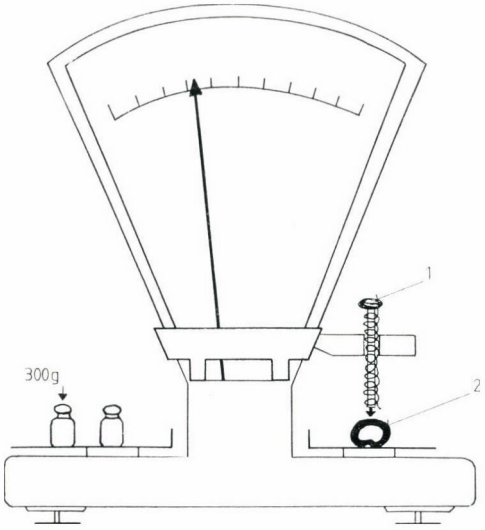

Fig. 1. Berkel scale balance showing the measurement of the softness of avocado after the combined treatment. The difference between the deflection of the needle (pointer) on the scale before and after the addition of the 300 g is an indication of the softness. 1: adjustable screw; 2: depression

and the weight at the time of inspection was expressed in percentage of the initial weight.

1.2.4.5. Internal appearance. — Randomly taken fruits were cut into half and observed for discolouration of the flesh. The internal appearance was rated as follows:

Rating	*Description*
5	excellent
4	good
3	acceptable
2	objectionable
1	unacceptable

1.2.4.6. Sensory evaluation. — The sensory attributes estimated were consistency and flavour of the flesh. A panel of eight judges scored the coded samples as follows:

Consistency:	*Rating*	*Description*
	6	extremely hard
	5	moderately firm
	4	slightly firm
	3	slightly soft
	2	moderately soft
	1	extremely soft

Flavour:	*Rating*	*Description*
	6	unacceptable
	5	objectionable
	4	slightly objectionable
	3	acceptable
	2	good
	1	excellent

1.2.4.7. Statistics. — The collected data were analysed using the standard analysis of variance and t test.

2. Results

1.2. Disease

The disease found on the avocadoes was mostly manifested by the dark brown to black discolouration on the skin, with fungal growth apparent at the later stage of rot (Fig. 2). As shown in Figs. 2 and 3, the combined treatment delayed and reduced the percentage of diseased fruits in comparison with the control, particularly on fruits stored first at 12 °C then moved to 20 °C.

2.2. Skin colour

Results are shown in Fig. 4. During post-shipment storage at 12 °C, the skin of the sound fruits remained green and there was no significant difference between the treatments. At 20 °C the non-irradiated fruits were slightly browner than the combined treated fruits.

2.3. Softness

Results obtained are shown in Fig. 5. From the results on fruits stored at 20 °C it would be noticed that throughout the storage period the irradiated fruits were significantly firmer indicating slower ripening. For fruits kept at 12 °C and then moved to 20 °C, no significant difference on softening was observed in whole fruits.

2.4. Weight loss

Results are shown in Fig. 6. No significant difference was observed between treatments. An increase in weight loss was noted when fruits were kept at higher temperature.

Fig. 2. Untreated (0 Gy) and combined treated (25 Gy) avocadoes at the termination of post-shipment storage (8 days at 12 °C plus 10 days at 20 °C). The packaging foil was removed immediately before the photo was taken

2.5. Internal appearance

Results in Table 1 show a clear difference in internal appearance between the two treatments. Throughout the storage period, the irradiated (combined

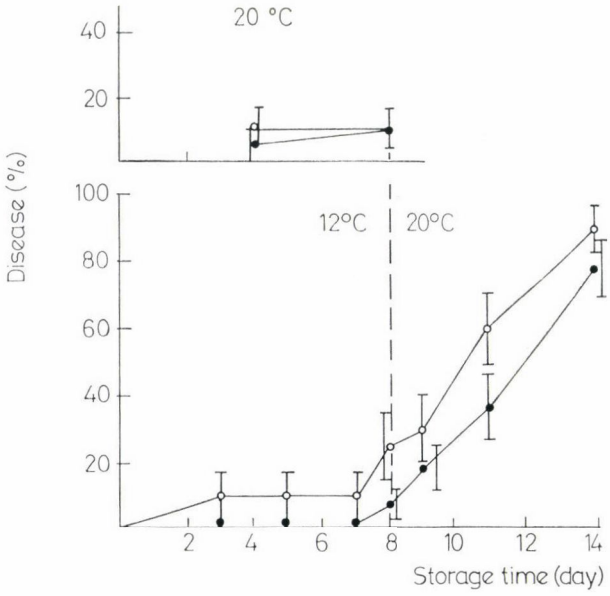

Fig. 3. Effect of combined treatment (10 min at 46 °C + 0.025 kGy) on decay of "Fuerte" avocadoes during post-shipment storage at 12 °C and 20 °C (mean values ± standard deviation), resp. ○ : non-treated; ● : treated

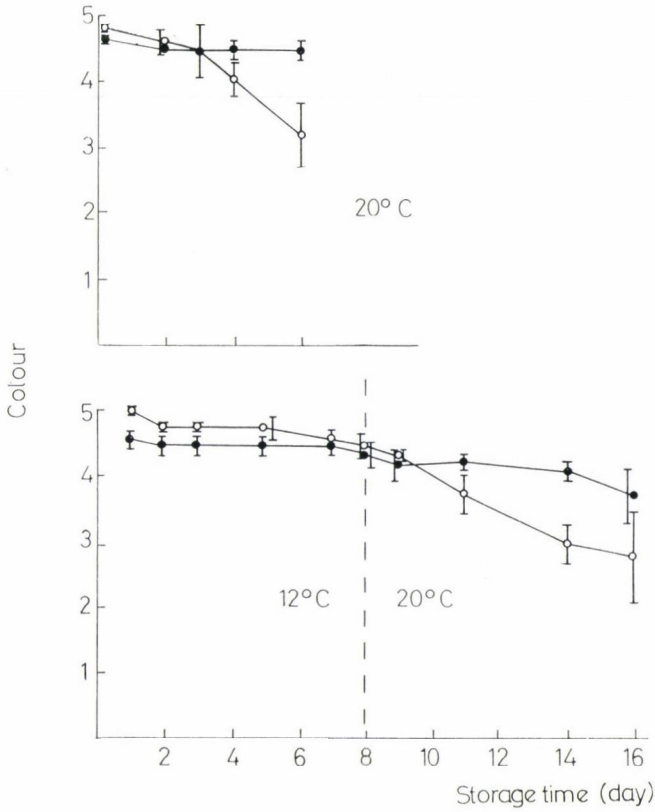

Fig. 4. Effect of combined treatment (10 min at 46 °C + 0.025 kGy) on the skin-colour of "Fuerte" avocadoes during post-shipment storage at 12 °C and 20 °C (mean values ± standard deviation), resp. ○: non-treated; ●: treated; 1: 100% brown; 2: 75% brown; 3: 50% green, 50% brown; 4: 75% green; 5: 100% green

treated) fruits were significantly better in appearance (Fig. 7). The non-treated fruits exhibited a brownish discolouration of the flesh which rendered them objectionable.

Table 1

Comparison of internal appearance of non-treated and combined treated Fuerte avocadoes during the post-shipment storage by t *test*

Days of storage	Non-treated		Treated	
	$\bar{x}$	±s	$\bar{x}$	±s
8 days at 20 °C	2.7	0.5	4.5*	0.03
8 days at 12 °C then 4 days at 20 °C	2.4	0.6	4.6*	0.3
8 days at 12 °C then 7 days at 20 °C	2.7	0.4	3.6	0.2
8 days at 12 °C then 10 days at 20 °C	2.4	0.7	4.2*	0.1

* Indicates that the difference between the treatments at that day of storage is significant at P = 0.05% probability level.

2.6. Sensory evaluation

Results are shown in Table 2. A significant difference in consistency was found on fruits kept for 4 days at 20 °C. The treated fruits were firmer than the non-treated fruits. Flavour, however, for both treatments was found to

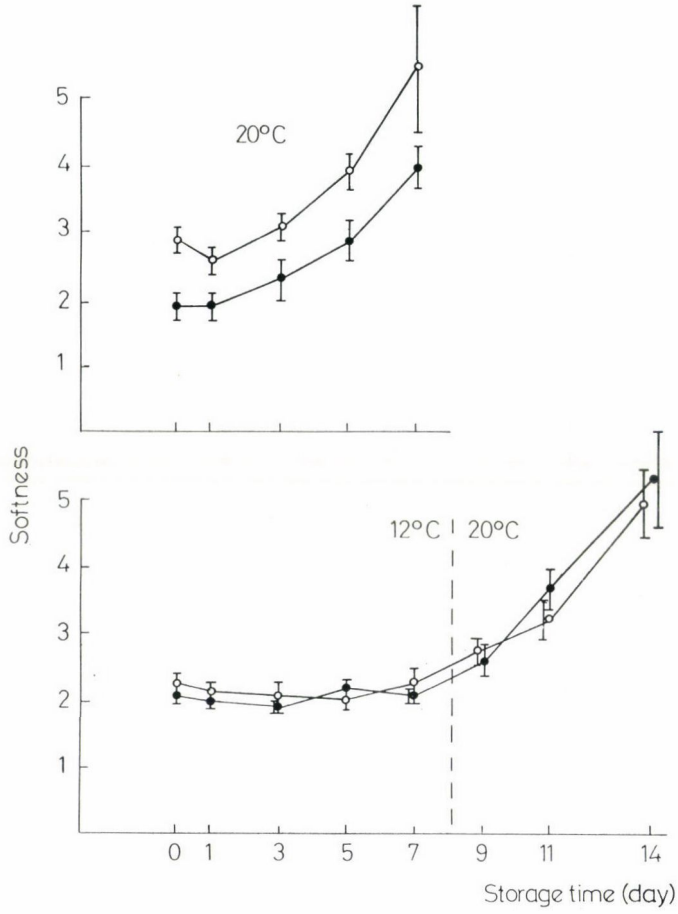

Fig. 5. Effect of combined treatment (10 min at 46 °C + 0.025 kGy) on the softness of "Fuerte" avocadoes during post-shipment storage at 12 °C and 20 °C (mean values ± standard deviation), resp. ○ : non-treated; ● : treated

be the same. On further storage at 20 °C for eight days, no difference in flavour and consistency was observed. Both had good flavour and slightly soft flesh consistency. For the fruits kept at 12 °C and then moved to 20 °C, generally the irradiated fruits were significantly better. Flesh consistency of the non-treated fruits was firmer but dry, and their flavour was objectionable.

Table 2

Comparison of sensory attributes (consistency of flesh and flavour) of non-treated and combined treated "Fuerte" avocadoes during the post-shipment storage by t *test*

Days of storage	Consistency of flesh				Flavour			
	Non-treated		Treated		Non-treated		Treated	
	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$
4 days at 20 °C	2.6*	0.3	4.1	0.3	2.3	0.2	1.1	0.3
8 days at 20 °C	2.7	0.4	3.1	0.5	2.4	0.2	2.3	0.3
8 days at 12 °C then 4 days at 20 °C	4.7*	0.3	2.4	0.3	4.6*	0.4	2.1	0.1
8 days at 12 °C then 7 days at 20 °C	3.9	1.4	1.4	0.2	4.2*	0.4	1.8	0.1
8 days at 12 °C then 10 days at 20 °C	2.2	0.4	1.9	0.3	5.1*	0.3	1.8	0.3

* Indicates that the difference between treatments at that day of storage is significan at P = 0.05% probability level

2.7. The role of wrapping

Wrapping the fruits in PVC foil has favourably influenced the keeping quality, probably by the modified atmospheric composition around the fruits. For that reason the frequent removal of the foil from the fruits for quality

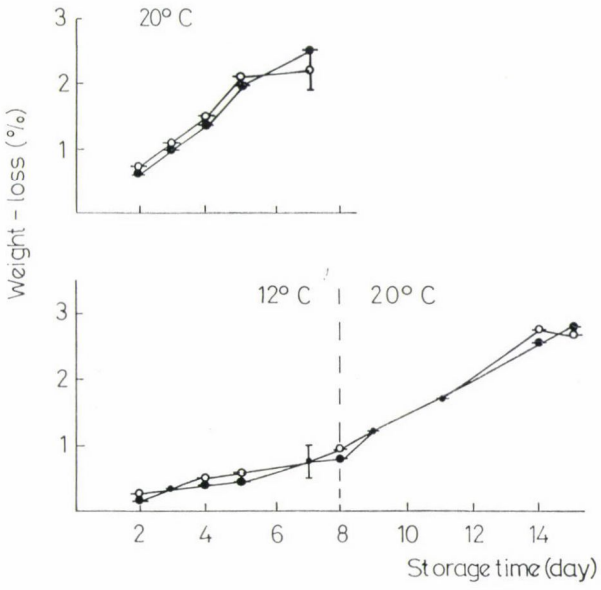

Fig. 6. Effect of combined treatment (10 min at 46 °C + 0.025 kGy) on the weight-loss of "Fuerte" avocadoes during post-shipment storage at 12 °C and 20 °C, resp. ○ : non-treated; ● : treated

Fig. 7. Outer and internal appearance of representative fruits after 8 days of post-shipment storage at 20 °C. (0 Gy = untreated fruits; 25 Gy = combined treated fruits). The packaging foil was removed immediately before the photo was taken.

Fig. 8. The effect of periodical inspection (unwrapping and handling) on the keeping quality of combined treated avocadoes. The left-hand side fruits have been kept wrapped during post-shipment storage for 8 days at 12 °C plus 7 days at 20 °C, and the wrapping was removed only immediately before the photo was taken. The right-hand side fruits have been unwrapped for inspection several times during the same period of storage.

inspection had an unfavourable effect, since the undisturbed avocadoes remained greener and showed less browning and decay (Fig. 8). However, the overall difference between untreated and combined-treated samples was similar in the undisturbed samples to that in those inspected repeatedly.

3. Conclusions

The aforementioned results support the previous South African and Chilean experiences and may allow the following conclusions:

— The combination treatment of mild heat (10 min at 46 °C) plus very low dose (0.025 kGy) irradiation delayed and reduced the decay of "Fuerte" avocadoes shipped from Chile to the Netherlands, and subsequently stored simulating distribution and retail display.

— The combination treatment and wrapping in PVC foil delayed senescence, resulting in less discolouration, better internal appearance, flesh consistency and flavour.

— This evaluation holds promise that a sea-transport of combined treated "Fuerte" avocadoes from Chile to Western-Europe would be feasible.

*

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ISOLATION AND EVALUATION OF PROTEIN FROM PROCESSED KARANJA *(PONGAMIA GLABRA)* SEED MEAL

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Attempts were made to prepare protein isolates from processed karanja seed meal (PKM) by extraction with salt solution or alkali solution alone or in combination and then precipitate with 10% trichloroacetic acid at acidic pH, centrifuge and freeze dry. Of the seven technologies adopted, extraction with 1 *M* NaCl solution yielded 10% protein product, 1 *M* Na_2CO_3 solution yielded 20% protein product, 5% $MgCl_2$ solution yielded 11% product, NaCl + $MgCl_2$ solution produced 12% and NaCl + $MgCl_2$ + Na_2CO_3 solution produced 12% while extraction with NaOH solution (pH 11) alone gave 15% product and a combined solution (pH 11) of NaCl, Na_2CO_3 and NaOH extracted 25% protein product. Of the salt or alkali solutions tried so far, the solution of Na_2CO_3 alone or in combination with other salt or alkali solutions was found most satisfactory for extraction of protein from the PKM. The protein contents of the products obtained by different technologies were also different with a maximum value of 88.7% (N×6.25). The present study demonstrated that by the seven technologies developed so far about 10–25% protein could be isolated from the 30% protein present in the PKM. The PKM-protein isolates were also nutritionally evaluated following well-established rat bioassay procedures in a comparative study with casein as standard. The results indicate that the PKM-protein isolates may be used as supplementary source of protein in animal feed.

Keywords: Processed karanja seed meal, seed protein isolate, nutritional evaluation

In recent years shortage and high prices paid for the traditional protein concentrates in livestock feeding had stimulated the interest in exploration of non-traditional seeds from forest plants as good sources of protein. Many a non-traditional waste seed may be utilized in animal feed provided they are properly processed and free from the toxic or antinutritional components (VIMAL & NAPHADE, 1980). In our earlier studies, it has been shown that defatted seed meals or seed protein of *Tictona grandias* (GHOSH MAJUMDAR et al., 1980), *Eucalyptus kirtoniana* (MANDAL et al., 1980; 1982a; 1984a), *Acacia auriculaeformis* (MANDAL et al., 1982b; 1984b) may be usefully exploited as supplementary source of protein and other nutrients.

Pongamia glabra (fam: Leguminosae), a medium sized glabrous tree, popularly known as karanja in India, is capable of growing under a wide range

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of agro-climatic conditions and is a common sight around coastal areas, river banks, tidal forests and roadsides in India and many other tropical countries. India has a potential to produce over one lakh (one hundred thousand) tonnes of karanja seed annually which is likely to increase. Recently, it has been identified as a potential new oilseed in different screening programs. The oil, which makes up roughly 30% of the seed weight, resembles groundnut oil in composition but contains a number of flavones (PARMAR et al., 1976) which seem to be primarily responsible for its toxicity (MANDAL, 1984). The oil is chiefly used for leather tanning, soap making, lubrication and in medicine. It is more suitable for these purposes after refining, since crude oil contains some furanoflavones which make it unsuitable for such uses. Recently, we have attempted to remove these toxic flavone compounds from the oil and it has been shown that the refined oil caused some metabolic abnormalities when consuming it for 12 weeks at a 10% diet level (MANDAL et al., 1984c) while refined and hydrogenated oil could be safely used up to the extent of 20% in the diet of albino rats (MANDAL et al., 1985). Being rich in oil which may be usefully exploited, the seeds of karanja may be of commercial importance.

Defatted karanja seed cake contains about 32–33% protein, 40% carbohydrates and other constituents, such as moisture, crude fibre, inorganic salts and unidentified vegetable matter. Besides, the seed meal contains karanjin [3-methoxyfurano-(2", 3" : 7, 8)-flavone; $C_{18}H_{12}O_4$; m. p. 158.5 °C], a toxic component, at a level of 0.35%, saponins at a level of 4%, a trace amount of a complex amino acid named glabrin (4,5 dihydroxy-N-methyl pipecolinic acid; $C_{21}H_{42}O_{12}N_3$; m.p. 290 °C] and free arachidic, lignoceric and behenic acids (COUNCIL OF SCIENTIFIC AND INDUSTRIAL RESEARCH, 1969). The feeding value of the seed meal is greatly impaired, however, by the presence of these undesirable components, though high protein content and its amino acid composition suggest that it might be a valuable supplement in animal feed (PARMAR et al., 1976; MANDAL et al., 1984b). During karanja processing studies in 1980, we observed that karanja seed meals prepared by conventional methods were toxic to rats and when seed meal was incorporated up to 30% in the diet of rats for 30 days, caused some metabolic and histopathological changes. On the contrary, in an earlier report, SINGH (1966) suggests that karanja cake can be used as a cheep cattle feed after oil extraction and detoxication. In 1984, we improved the palatability and reduced toxicity of karanja seed meal by hydrolysis of the seed meal with 2% hydrochloric acid for 5 h (MANDAL et al., 1984d), wherby the toxic components were destroyed. In this detoxication process, toxic saponins were converted into non-toxic sapogenin by acid hydrolysis and karanjin, glabrin and other antinutritional factors were decomposed by the action of moist heat under basic conditions. Although, this process was satisfactory for yielding a non-toxic

seed meal supplement for ruminant animals (MANDAL et al., 1984d), the protein quality of the processed seed meal was not assessed. Even the technology for the isolation of the protein from the processed seed meal needs to be investigated.

In the present study, attempts have been made to prepare protein isolate from the processed karanja seed meal and the protein quality has been nutritionally evaluated in feeding trials on albino rats. Food intakes, growth response and some biological indices were measured to assess the nutritive value of the isolated proteins.

1. Materials and methods

Pongamia glabra (karanja) seeds were collected from the local forests of Burdwan, West Bengal, India and completely powdered. The powdered seeds were completely deoiled by solvent (n-hexane) extraction method in a Soxhlet distillation apparatus for 72 h and air dried to remove the traces of solvent.

1.1. Processing of the seed meal

Deoiled seed cake was processed following the same method as described earlier (MANDAL et al., 1984d). In this method about 500 g of the seed meal was refluxed for 5 h with 2 dm^3 of 2% hydrochloric acid. The mixture was then cooled and about 45 g of lime dissolved in 100 cm^3 of water was added. The slurry was stirred for 1 h and then dried on a hot plate. The processed seed meal (PKM) was stored in cold for the isolation of protein.

1.2. Methods adopted for the isolation of protein from the PKM

The processed karanja seed meal (PKM) was used as raw material which contains approximately 30% of crude protein (N × 6.25). The PKM was extracted with different salt or alkali solutions alone or in combination and the extract was separated from the fibrous product by centrifugation and it was then filtered. Protein was precipitated in the extract by adding 10% trichloroacetic acid (TCA) at acidic pH and after washing with water the protein was finally collected by centrifugation and freeze dried. All technological processes were repeated five times.

1.2.1. Salt extraction technology (T 1). About 100 g of the PKM was suspended in 1 dm^3 of 1 *M* NaCl solution and stirred for 1 h. The slurry was centrifuged (5000 g for 20 min) and the supernatant fraction was collected after filtration. The residue was washed once with 500 cm^3 of 1 *M* NaCl solution and the supernatant fraction collected after centrifugation and filtered.

Protein in the combined supernatant fractions was precipitated at pH 3.4 by adding 10% TCA. After sedimentation, the sediment was separated by decantation and finally the protein was separated by centrifugation and freeze dried.

1.2.2. Salt extraction technology (T 2). This technology was identical with that of salt extraction technology T 1, but 1 *M* Na_2CO_3 solution was used instead of 1 *M* NaCl for the extraction of protein from the PKM.

1.2.3. Salt extraction technology (T 3). This technology was also similar to the above salt extraction technologies. The only difference was that a 5% solution of $MgCl_2$ was used for the extraction of protein from the PKM.

1.2.4. Mixed salt extraction technology (T 4). About 100 g of the PKM was suspended in 1 dm^3 of water and 20 g of NaCl and 30 g of $MgCl_2$ were added. The suspension was stirred for 1 h and centrifuged. The residue was washed once with 500 cm^3 of 1 *M* NaCl solution and the supernatant collected after centrifugation. Protein in the combined supernatants was precipitated at pH 3.4. The sediment was decanted, centrifuged and freeze dried.

1.2.5. *Mixed salt extraction technology (T 5).* About 100 g of the PKM was suspended in 1 dm^3 of water and a salt mixture containing 20 g of NaCl, 30 g of $MgCl_2$ and 25 g of Na_2CO_3 was added. The slurry was stirred, centrifuged and filtered. The residue was washed with 500 cm^3 of 1 *M* NaCl solution and the supernatant was collected after centrifugation. Precipitation and collection of the protein product was done following the above mentioned procedure.

1.2.6. Basic technology (T 6). About 100 g of the PKM was suspended in 1 dm^3 of 0.25% Na_2SO_3 solution (Na_2SO_3 was used as antioxidant). The pH of the suspension was adjusted to 11 by adding 5 *M* NaOH solution. The slurry was stirred, centrifuged and the supernatant was collected after filtration. The residue was washed once with dilute NaOH solution (pH 11) and the supernatant was collected after centrifugation. Protein was precipitated in the combined supernatant fractions at pH 4.2 by adding 10% TCA and collected following the same method as stated above.

1.2.7. Mixed salt basic technology (T 7). About 100 g of the PKM was suspended in 1 dm^3 of water and 20 g of NaCl and 25 g of Na_2CO_3 were added. The pH of the suspension was adjusted to 11 by adding 5 *M* NaOH solution. Extraction, precipitation and collection of the protein product was done following the same method as mentioned above.

1.3. Analytical methods

1.3.1. Moisture content. Moisture content of the protein sample was determined by direct heating the sample at 105 $\pm$ 2 °C as described by RAGHURAMULU and co-workers (1983).

1.3.2. Ash content. A protein sample was taken in a platinum crucible and heated in a muffle furnace for about 3–4 h at about 600 °C. It was then

cooled in a desiccator and weighed. The process of heating and cooling is repeated till a constant weight is achieved. The procedure followed was as described by RAGHURAMULU and co-workers (1983).

1.3.3. Nitrogen content. Total N content of the protein isolates were determined by micro-Kjeldahl analysis following the method as described by RAGHURAMULU and co-workers (1983).

1.3.4. Soluble protein content. Five mg of the protein isolate was extracted. with 200 cm^3 distilled water for 1 h on a shaking apparatus at room temperature. The extract was centrifuged and the liquid filtered. The residue was again extracted with 100 cm^3 distilled water, centrifuged and the liquid filtered. The centrifuged and filtered liquids were combined and their protein content ($N \times 6.25$) was determined by micro-Kjeldahl analysis.

All analyses were replicated and accepted with a deviation from the mean value not more than 5%.

1.4. Nutritional evaluation of the PKM-protein isolates

Protein isolates of PKM obtained by different methods were nutritionally evaluated in feeding trials with albino rats.

1.4.1. Animals and their diets. The animals used for the feeding experiments were male albino rats of local strain (inbred in our laboratory) weighing about 45–50 g. The animals were individually caged and maintained under controlled temperature (21 ± 1 °C) and humidity (55%) conditions. The animals were distributed, six per group, equalized for body weight. One group was fed 10% casein based diet and seven others were assigned to diets containing 10% protein isolate of PKM obtained by different methods (one group for each type of protein-isolate). The composition of the protein-free basal diet was similar to that used earlier by us (MANDAL et al., 1982a). The protein-free basal diet was mixed with casein or with the test protein isolate to provide 10% protein diet for the experimental animals. All the animals in different groups received their diets and water ad libitum for 28 days. Daily food consumption and body weight were determined for each animal.

1.4.2. Nutritional indices. Protein efficiency ratio (PER) was calculated from values for body weight gain and CP (N content of the test diet $\times 6.25$) intake over a 28-day period. PER was calculated as body weight gain per unit CP intake. True digestibility (TD) and biological value (BV) were calculated from information on carcass composition with a protein-free and the test diet. The procedure followed was as described by SWAMINATHAN (1981). Metabolic and endogeneous N were determined separately with a protein-free diet (with four rats) and then mean values were used in the determination of BV and TD.

2. Results

2.1. Protein isolates and their properties

The amounts of protein products of PKM obtained by different methods and their properties are shown in Table 1.

It is evident from the results that protein isolated by different methods differ with regard to a variety of properties, even appreciable differences in the efficiencies of the individual technologies were found. The amount of protein product obtained in different salt extraction technologies were different, it was 10% when 1 *M* NaCl solution was used as extraction solution, 11% when 5% $MgCl_2$ solution was used and 20% when 1 *M* Na_2CO_3 solution was employed. The protein isolate obtained by mixed salt technology (T 4) was 12%, 21% in T 5 method while basic technology (T 6) produced 15% and mixed salt basic technology (T 7) gave 25% protein product. Total nitrogen (N) content of the protein isolates varied within the range of 12.4 to 14.2%. Solubility of the protein isolates in water differs significantly with a

Table 1

Properties of the protein isolates of PKM by different methods

Sign of technology	Technology	Protein precipitation		Drying method	Protein content[a] (%)
T 1	Extraction with NaCl solution (pH 7.2)	Acidic protein precipitation	pH 3.4	Freeze-dried	10
T 2	Extraction with Na_2CO_3 solution (pH 10.4)		pH 3.8		20
T 3	Extraction with $MgCl^2$ solution (pH 7.0)		pH 3.4		11
T 4	Extraction with mixed salt (NaCl + $MgCl_2$) solution (pH 7.1)		pH 3.4		12
T 5	Extraction with mixed salt (NaCl + $MgCl_2$ + + Na_2CO_3) solution (pH 10.2)		pH 3.8		21
T 6	Extraction with NaOH solution (pH 11)		pH 4.2		15
T 7	Extraction with mixed salt (NaCl + Na_2CO_3) basic (NaOH) solution (pH 11)		pH 4.2		25

Sign of technology	Loss during isolation[a] (%)	Protein soluble[b] (%)	Total N content[b] (%)	Moisture[b] (%)	Ash[b] (%)	Visual colour
T 1	20	6	12.4	6	1.8	brown
T 2	10	8	13.8	4	2.1	light brown
T 3	19	4	12.6	6	1.6	brown
T 4	18	5	12.8	7	1.6	brown
T 5	9	8	14.1	5	2.0	white
T 6	15	10	13.8	6	1.9	light brown
T 7	5	12	14.2	5	2.2	white

[a] Percentage of the seed meal
[b] Percentage of the protein isolate
T 1 to T 7: for legend and symbols see Materials and methods

maximum value of 12% for the protein isolate obtained by mixed salt basic technology (T 7). There are no marked differences in moisture and ash content of the products obtained by different methods. The colour of the products varied from brown to white and they were devoid of unpleasant odour and taste.

2.2. Nutritional performances of the PKM-protein isolates

Nutritional and biological indices for rats given diets containing casein and PKM-protein isolates for 28 days, are presented in Table 2.

Table 2

Nutritional and biological indices for rats given diets containing casein and PKM-protein isolates

Protein source		Body weight gain (g per 28 days)		CP (N×6.25) intake (g per 28 days)		PER		TD		BV	
		$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s
Casein		68.1	2.1	24.6	1.5	2.77	0.21	0.91	0.01	0.88	0.01
PKM-protein isolate	T 1	39.7	1.6	24.8	1.3	1.60	0.18	0.82	0.02	0.49	0.02
	T 2	40.6	1.4	24.6	1.4	1.65	0.16	0.84	0.01	0.51	0.04
	T 3	40.9	1.8	24.8	1.2	1.65	0.18	0.80	0.04	0.50	0.02
	T 4	40.3	1.9	24.9	1.1	1.62	0.16	0.81	0.03	0.52	0.01
	T 5	41.5	1.5	24.7	1.6	1.68	0.19	0.90	0.01	0.50	0.04
	T 6	39.6	2.1	24.8	1.5	1.60	0.16	0.86	0.02	0.51	0.03
	T 7	40.5	1.4	24.7	1.4	1.64	0.20	0.90	0.01	0.52	0.05

$\bar{x}$: mean value from six rats; ±s: standard deviation; CP: crude protein; PER: protein efficiency ratio; TD: true digestibility; BV: biological value; T 1 to T 7: for legend and symbols see Materials and methods

The results suggest that body weight gain and CP intakes were almost similar in different groups of rats fed diets containing PKM-protein isolates but the values were lower than those fed diet containing casein. The results recorded with feeding trials with rats show that inclusion of 10%-protein products in the diets, caused the PER and BV values to be in the range of 1.60–1.68 and 0.49–0.52, respectively, compared with that recorded for casein (PER 2.77 and BV 0.88). True digestibility of the PKM-protein isolates varied in the range of 0.80–0.90 and compared well with that of casein (0.91).

3. Conclusions

3.1. Comparison of the methods for the isolation of protein from the PKM

Efficiencies of the individual technologies for the isolation of protein from PKM were markedly different. Of the salt (NaCl, $MgCl_2$, Na_2CO_3, NaCl + + $MgCl_2$, NaCl + $MgCl_2$ + Na_2CO_3) solutions, alkali (NaOH) solution and

mixed salt-alkali (NaCl + Na_2CO_3 + NaOH) solution tried so far, Na_2CO_3 solution alone or mixed with other salt or alkali solution was found to be most satisfactory for extraction of protein from the PKM. The highest amount of product was obtained in the mixed basic technology (T 7) along with the highest amount of protein (88.7) in that isolate. It was also found that when the PKM-protein isolates were washed with 96% ethyl alcohol at room temperature for 15 min under blending, the protein yield increased by 5–8%. Alcohol washing also reduced the visible colour and unpleasant odour if any. It is evident that during the preparation of the protein isolates under the experimental conditions of this study, approximately 5–20% protein was lost. The losses were lowest with those methods in which Na_2CO_3 solution alone or mixed with other salt or alkali solutions was used. Thus, it appears that Na_2CO_3 solution alone or in combination with another salt or alkali solution is the best solvent for the extraction of protein from PKM.

3.2. Possibility of PKM-protein isolates for nutritional exploitation

The results of this short feeding study with rats show that PKM-protein isolates in the diets for 28 days resulted in body weight gain which was about 60% of that with casein diet. The PER value of the seed protein isolates varied within the range of 1.60 to 1.68 and the BV value in the range of 0.49 to 0.52. The results revealed that these values were lower than those obtained for casein but at the same time they compared well with those of some standard edible vegetable proteins such as coconut protein (PER, 2.0 and BV, 0.67), cottonseed protein (PER, 2.1 and BV 0.63), groundnut protein (PER, 1.7 and BV, 0.54), maize protein (PER, 1.2 and BV, 0.59), soybean protein (PER, 1.6 and BV, 0.49) and sesame protein (PER, 1.7 and BV, 0.60) (Drouliscos & Malefaki, 1980; Gopalan et al., 1984). The digestibility of the seed protein isolates was almost similar to that of casein. Thus, it may be concluded that the PKM-protein isolate may be usefully exploited as a supplementary source of protein in animal feed. However, this seed protein isolate needs to be evaluated in a long-term toxicological study in different test animals by a multigeneration breeding technique before it can be recommended as safe for animal consumption. Such studies are in progress in our laboratories.

*

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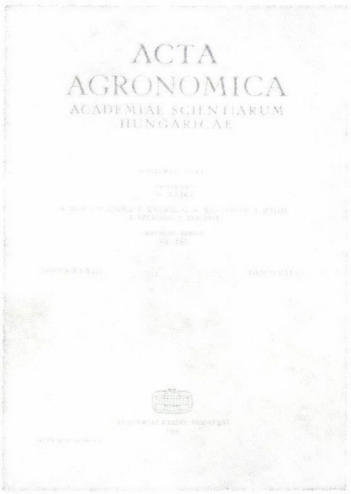

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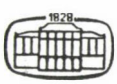

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LOW TEMPERATURE FLUIDIZED-BED DRYING OF MUSHROOM, CARROT, BEEF AND SHRIMP SAMPLES

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A drying technique at low temperatures in a fluidized-bed of activated alumina particles is described. The technique was used to test dry samples of mushroom, carrot, beef and shrimps. The influence of sample dimensions and fluidized-bed temperature on drying time were investigated. A drying regime was developed combining freeze-drying and air-drying operations to produce completely dried and shelf-stable food samples. The quality of these products were compared to that of analogues either completely freeze-dried in vacuo or dried partially by vacuum freeze-drying followed by air-drying. Products dried by the fluidized-bed technique were judged to be of good quality. The use of the atmospheric fluidized-bed drier is projected to result in the production of less expensive dried food samples.

Keywords: fluidized-bed drying, mushroom, carrot, beef, shrimp

Freeze-drying reduces food weight with little loss of initial quality. However, because of their cost, the market for freeze-dried foods tends to be limited somewhat to a special clientele comprising among a few others campers, military and space programmes (LONGAN, 1973). Furthermore, techniques of compression and plasticization were developed (MACKENZIE & LUYET, 1969; WISAKOWSKY, 1977) to reduce the volume of freeze-dried foods in order to save space and thereby reduce packaging and transportation costs.

It has been demonstrated by SCHADLE and co-workers (1983) that it is possible to dry foods by combining freeze drying and hot air-drying to yield products with qualities comparable to completely freeze dried samples. The same workers applied temperatures as high as 60 °C to complete drying after the initial freeze-drying stage. As noted by FLINK (1977), energy requirements per unit weight of water removed during drying is greater for freeze-drying than for air-drying. Ultimately therefore, a combination of freeze-drying and air-drying would achieve a reduction in total energy requirements.

It is thermodynamically possible to conduct a freeze-drying operation at pressures obtainable in an air-drier. By combining freeze-drying and air-drying operations in one equipment operating under atmospheric conditions, a further reduction in costs is expected primarily as a result of the suppression of vacuum creating components. However, techniques that can successfully realize the sublimation of ice under atmospheric conditions, must satisfy the fundamental

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requirement that a partial vapour pressure of water less in magnitude than the triple point vapour pressure of ice, about 610.6 Pa at 0 °C, be maintained in the drying air. The mass transfer potential for freeze-drying is then provided by the partial vapour pressure differential between the ice in the food sample and the surrounding air.

Fluidized-bed systems for drying solids have been in existence for a long time. They have been used extensively in the chemical industry particularly for drying materials such as limestone, coal, cement. Economic considerations made these units attractive especially when large quantities of material were to be dried. From a kinetic view point, an important feature of fluidized-bed systems is their high heat transfer rates. These could be 5 to 25 times greater than heat transfer rates for the drying gas (air) alone. Another kinetically important characteristic of fluidized-bed systems is that, except for columns with extreme length to diameter ratios, temperature is by and large uniform at all points of the fluidized-bed (KUNNII & LEVENSPIEL, 1969; PERRY & CHILTON, 1973).

In the food industry, fluidized-bed systems have been developed for coffee roasting (ANDRADE AISPURO, 1981). Fluidized-bed systems have also been used in freezing operations involving foods with granular geometry. VAZQUEZ and CALVELO (1983) reported that fluidized bed freezers were used to produce individually quick frozen (IQF) peas, potato chips and diced potatoes. MINK and SACHSEL (1968), DRYDEN and NACK (1966), SACHSEL and MINK (1967) applied fluidization to the freeze-drying of food products in vacuo; MALECKI (1967), MALECKI and co-workers (1970), GIBERT (1977), WACHET (1978), BOEH-OCANSEY (1979, 1983, 1984) among several researchers have attempted to use fluidized-bed systems to freeze-dry food products under atmospheric conditions.

In this work, a drying technique at low temperatures in a fluidized-bed medium of activated alumina particles was developed. In a previous paper (BOEH-OCANSEY, 1983) a study was reported of the use of this technique to freeze-dry liquid foods. The influence of concentration or dry matter content on the drying mechanism was investigated. The study was designed to provide information on what to expect when the technique was applied to the freeze-drying of other foods differing principally in initial moisture content. BOEH-OCANSEY (1985) reported the application of the atmospheric technique to the freeze-drying of a solid texturous foodstuff, namely carrot. The effects of product dimensions and fluidized-bed temperature on freeze-drying time were discussed but no attempt was made to obtain completely air-dried food samples.

The over-riding objective of this study is to develop a drying procedure which would combine in the same equipment the advantages of freeze-drying and hot air-drying as well as those of fluidized-bed systems operating at atmospheric pressure. This way, it is hoped that less expensive dried foods of very

good quality and competitive with freeze-dried products could be produced. In this paper, the drying patterns for four food types namely carrot, mushroom, beef and shrimps are presented and a methodology for producing completely air-dried samples is developed. The temperature programming technique developed effectively combined in one equipment operating under atmospheric conditions, freeze-drying and air-drying operations. Drying periods were thus curtailed. The quality of representative samples dried by the technique were compared to that of completely vacuum freeze-dried samples and also to partially vacuum freeze-dried then air-dried analogues.

1. Materials and methods

1.1. Preparation of food samples

Four food types namely, mushroom, carrot, beef and shrimp of respectively 91.2 %, 89.5 %, 70.6 % and 82.5 % moisture content (wet weight basis) were used for this study. Decorticated, precooked shrimps, beef (rumpsteak) and mushrooms (champignon de Paris) were obtained commercially quick-frozen. The mushrooms and beef were cut into strips while the shrimps were cut into cylindrical pieces. Carrots, on the other hand, were purchased raw fresh; they were cut into disc-shaped units which were then frozen at −35 °C for at least 8 h. The dimensions of the cut food pieces were measured. The samples were sorted and their weights recorded. The measured characteristics of the food sample units are assembled in Table 1. All samples were left in frozen storage at −35 °C until they were required for drying.

1.2. Fluidized-bed drier

A general diagram of the fluidized-bed drier is presented schematically in Fig. 1. The drier consists principally of a cylindrical column, a refrigeration unit and an air blower. The drying chamber is a vertical cylindrical column 0.10 m in diameter, 1.00 m high and thermally insulated. The drying column contains 1 kg of dry activated alumina particles of 0.4×10^{-4} m average diameter into which the food samples are immersed. The column is joined below to a similarly-shaped arrangement linked to the refrigeration unit and thereby constituting a heat exchanger. Air is supplied to the drying column by a blower Fougal HD40A that yields a maximum volumetric flow rate of 0.2 $m^3 s^{-1}$ at 20 °C and a corresponding discharge pressure of 1333.2 Pa. A maximum discharge pressure of 5866.2 Pa is obtainable, however, but at the reduced volumetric flow rate of 0.04 $m^3 s^{-1}$ at 20 °C.

Air temperature was measured at various locations of the pilot plant drier by the use of copper-constantan thermocouple probes connected to a multi-

Table 1

Physical characteristics of food samples

Characteristics[a]	Mushroom		Shrimp			Carrot		Beef
Shape	Block-shaped strips		Cylindrical			Discs		Strips
	A	*B*	*A*	*B*	*C*	*A*	*B*	
Dimensions ($m \times 10^{-3}$)	5×9×12	7×11×15	(diameter/length ratio) 9/13	10/13	10 (maximum diameter)	(diameter/thickness ratio) 20/6	22/4	7×10×13
Average weight of unit ($kg \times 10^{-3}$)	0.4	0.7	0.5	0.8	1.5	1.8	2.0	0.5

[a] Samples were sorted such that: dimensions ± 1 mm, weight ± 0.1 g

channel potentiometric recorder. The desired drying temperature was obtained by the judicious mixture of cold air from the cooling section with warm recycled air coming directly from the air blower through the by-pass arrangement as shown in Fig. 1. Constant cold drying temperatures of about −10 °C and less were however maintained by direct thermostatic control of the refrigeration unit. Air flow rates were measured using differential water manometers connected to venturis, while air humidity was monitored by an electrolytic hygrometric recorder (Beckman, USA).

In the fluidized bed, drying of immersed food products is obtained as the fluidizing alumina grains adsorb moisture directly from the food samples upon contact and indirectly by capturing moisture in the air. The adsorbent particles maintain a water vapour pressure differential between the air and food product and thus ensure the transport of moisture from the food samples to the air. The superficial air velocity during drying was maintained at a rate one and one-half times the minimum fluidizing velocity of the activated alumina grains. At this flow rate the bed exhibited a state of good fluidization. At all times during drying the relative humidity of the air was maintained at values below 20 ppm (2×10^{-5} kg moisture per kg dry air).

At the end of drying, recovery of immersed food products was achieved by the use of a perforated cylindrical container (sieve) placed inside the drying chamber in a way that does not hinder the free circulation of the fluidizing medium. The cylindrical sieve was fixed to the cap of the drying chamber. Drying was accompanied with the monitoring of changes in the weight of

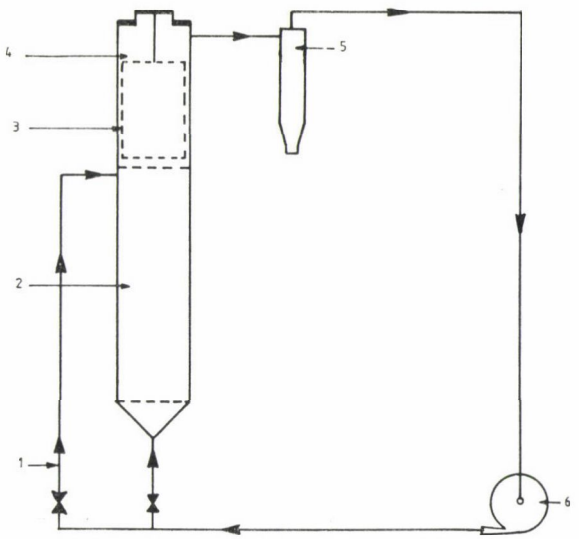

Fig. 1. Fluidized-bed drier. 1: by-pass; 2: cooling section; 3: perforated cylindrical container; 4: drying chamber; 5: cyclone; 6: air blower

0.050 kg and 0.20 kg sample batches with time. At the end of each drying operation, used alumina was regenerated by heat treatment at 200 °C for 30 minutes. The regenerated alumina particles were thereafter left to cool in hermetically sealed jars before reuse.

1.3. Moisture content determination

Ten grammes of food sample were weighed into a clean, dry aluminium dish. The container was then placed in a vacuum oven at a fixed temperature of 64 ± 1 °C for at least 5 h; the operating pressure was 3 kPa. The difference in sample weight, before and after drying, is recorded and is used to compute sample's moisture content either on wet weight basis or on dry weight basis.

2. Results

2.1. Characteristics of the fluidized-bed

The minimum fluidizing velocity of the alumina granules (4×10^{-4}m diameter) was 0.11 m s^{-1}. Preliminary investigations demonstrated that immersion of extraneous objects of the dimensions and shapes of the food particles into the fluidizing bed had little effect on the bed's fluidization behaviour. Slight increases in pressure drop were however observed across the fluidized-bed.

Figure 2 presents the adsorption isotherm of alumina grains for water at 25 °C, illustrating the quantity of moisture adsorbed by 0.10 kg of alumina

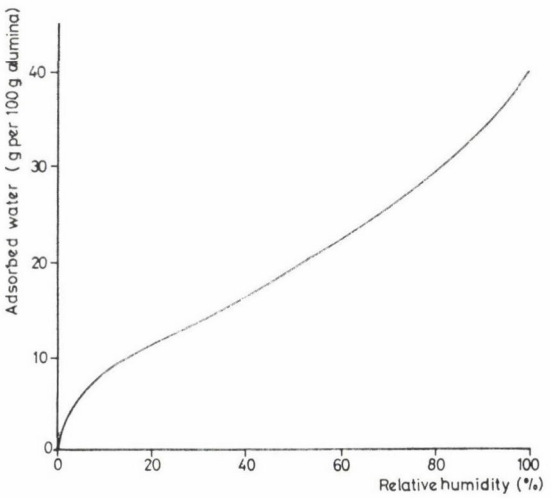

Fig. 2. Adsorption isotherm of alumina granules for water at 25 °C

grains while the relative humidity of the air is varied. Other physical properties of the alumina grains employed in the fluidizing medium (ANON., 1977) are:

- a specific surface area in excess of 3.45×10^5 m^2 kg^{-1};
- a volume of micropores estimated at 4.00×10^{-4} m^3 kg^{-1};
- an apparent density of 770 kg m^{-3}; and
- a specific density of 30 kg m^{-3}.

2.2. *Drying curves*

Drying curves are presented for the given food samples in Figs. 3 through 7. The curves were obtained by plotting the mass fraction of removed moisture ($m_0 - m$ per m_0) against drying time; m_0 represents the initial moisture content of the sample and m the moisture content after t hours of drying.

A visual inspection of Figs. 3 and 4 reveals that sample dimensions and bed temperature are important factors that influence the drying kinetics. For instance, after 7 h of drying at −5°C, mushroom sample *A* having di-

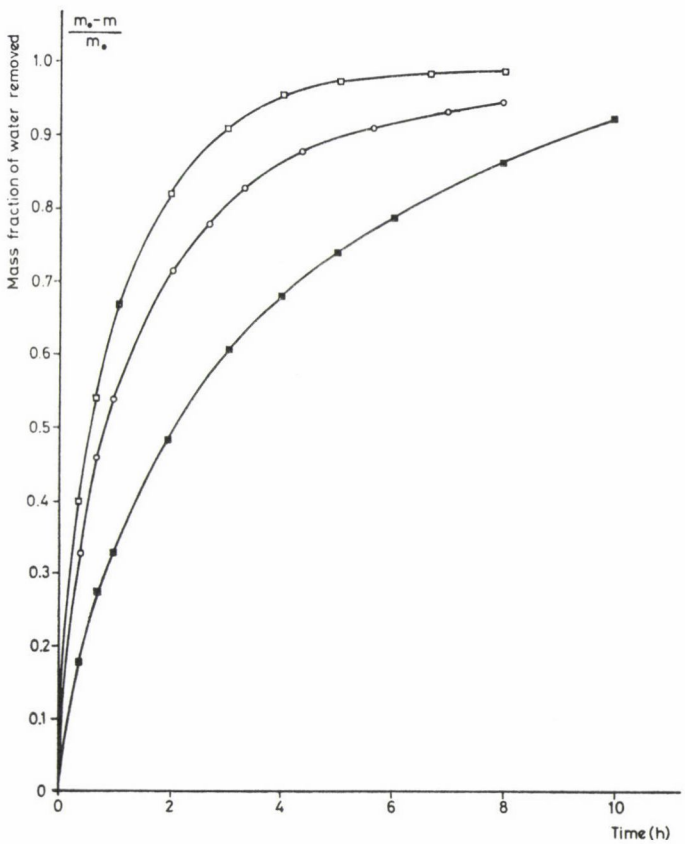

Fig. 3. Constant temperature drying of mushroom. □: sample *A*, −5 °C; ■: sample *A*, −15 °C; ○: sample *B*, −5 °C

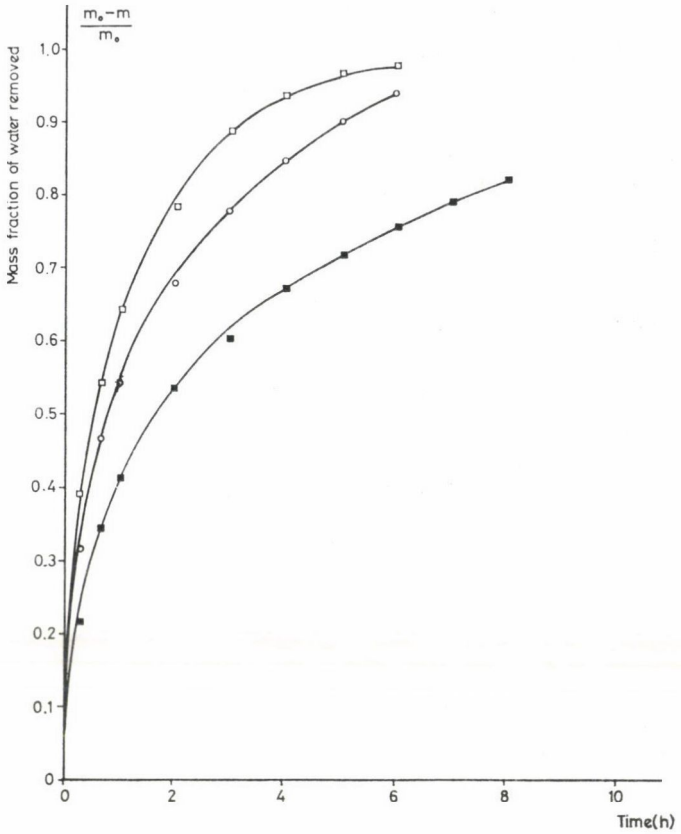

Fig. 4. Constant temperature drying of shrimps. □: sample *A*, −5 °C; ■: sample *A*, −20 °C; ○: sample *B*, −5 °C

mensions of 5×9×12 mm attained a moisture content of 13.5% (wet weight basis) while the bigger sample *B* (7×11×15, 10^{-3} m units) retained almost 3 times as much moisture; its moisture content was 40.3% (Fig. 3). Similarly, after 5 h of drying shrimps at −5 °C (Fig. 4), shrimp sample *A* with a diameter-to-length ratio of 9 per 13 (Table 1) attained a moisture content of 13.5% whereas sample *B* (diameter–length ratio 10 : 13) retained as much as 31.4% moisture.

In addition, it was generally observed that the lower the fluidized-bed temperature, the lower the drying rate and consequently the longer the drying time. Referring again to Fig. 3, 90% of the total moisture in mushroom sample *A* was removed within 3 h of drying at −5 °C whereas about 10 h were required to remove the same quantity of moisture from another batch of the same sample at −15 °C. Similarly, with reference to shrimp drying at −5 °C

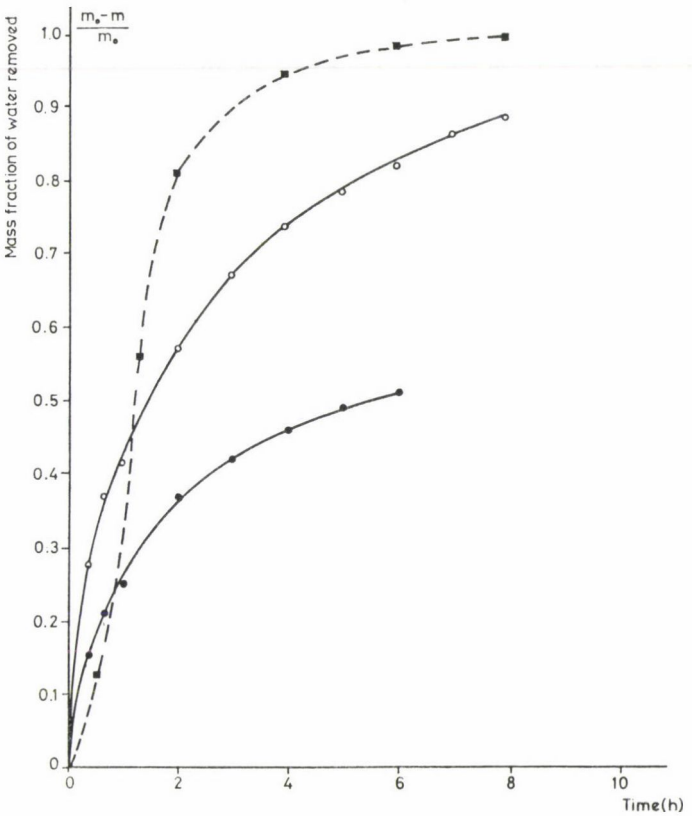

Fig. 5. Drying of beef samples in a vacuum dryer and in the fluidized-beddryer. ■: vacuum drying: ○: fluidized-bed drying, −5 °C; ●: fluidized-bed drying, −20 °C

(Fig. 4), 80% total moisture was removed from sample *A* (Table 1) within 2.5 h; at −20 °C, removal of the same quantity of moisture from a second batch of the same sample took about 9 h.

In addition to presenting drying curves for beef (rumpsteak) at two temperatures, −5 °C and −20 °C, Fig. 5 also compares these with a drying curve for the same product where a laboratory-scale vacuum dryer (Serail RP3V) was used. After 5 h of drying in the atmospheric fluidized-bed dryer, 42% of total moisture had been removed at −20 °C; at −5 °C, 66.5% total moisture was removed during the same period. Using the vacuum dryer 89% total moisture was removed within the same time. To obtain completely dried samples, 9 hours of vacuum drying were required; however, at least 14 h drying at −5 °C was deemed necessary to obtain completely dried samples using the fluidized-bed dryer; at −20 °C the drying would have to last days.

3. Conclusions

The design of any fluidized-bed system requires detailed information on solid fluidization conditions, on gas particle heat transfer coefficients and on residence time of solids in the fluidizing medium. Fluidization conditions are influenced by the superficial velocity of the gas (air) as well as by the size and shape of the particles that constitute the fluidizing medium. The thermal properties of the gas (air) and its superficial velocity are factors which affect the heat transfer coefficients of fluidizing beds. The density of solids in the fluidizing bed and the size and shape of these particles are equally important parameters that also influence the heat transfer coefficients of fluidized-bed systems.

Activated alumina exhibits a high adsorption capacity per unit weight as well as a very high specific surface area. These physical properties result from its extremely high porosity. Even when saturated activated alumina grains appear in all respects dry.

The slight increases in pressure drop observed across the fluidized bed when extraneous objects were immersed in the bed were found to be proportional to the total volume of immersed objects. These increases were correspondingly in direct proportion to the accompanying increase in the bed height observed, as Ergun's equation predicts (KUNNII & LEVENSPIEL, 1969; DAVIDSON & HARRISON, 1971).

The heat and mass transfer characteristics of fluidized beds of alumina grains were studied by WACHET (1978), GIBERT (1968).

Compared to the vacuum process, longer periods were required to completely dry food samples by the atmospheric fluidized-bed technique. However, at temperatures above 0 °C, the fluidized-bed dryer took shorter periods to dry the samples. The quality of such products was however, markedly inferior to that of analogues dried at lower temperatures. These samples manifested marked shrinkage, poor colour and, on the whole, poor general appearance. On the other hand, drying at temperatures below −5 °C yielded good quality products but required very long drying periods. In addition, technico-commercial considerations would require that food sample units be somewhat bigger than the mushroom and beef strips as well as the shrimp cylinder (Table 1) used for the study. Experimentation was consequently continued using larger shrimp cylinders (sample C) whose average unit weight of 1.5×10^{-3} kg was three times that of sample A and almost double that of sample B. Carrot discs with average unit weights of up to 2.0×10^{-3} kg were also used (Table 1). Very long periods were indeed required for drying such samples at low temperatures. For example, at a fluidized-bed temperature of −10 °C, periods by far exceeding 20 h would be required to dry 4 mm-thick sample B carrot discs as can be estimated from Fig. 6.

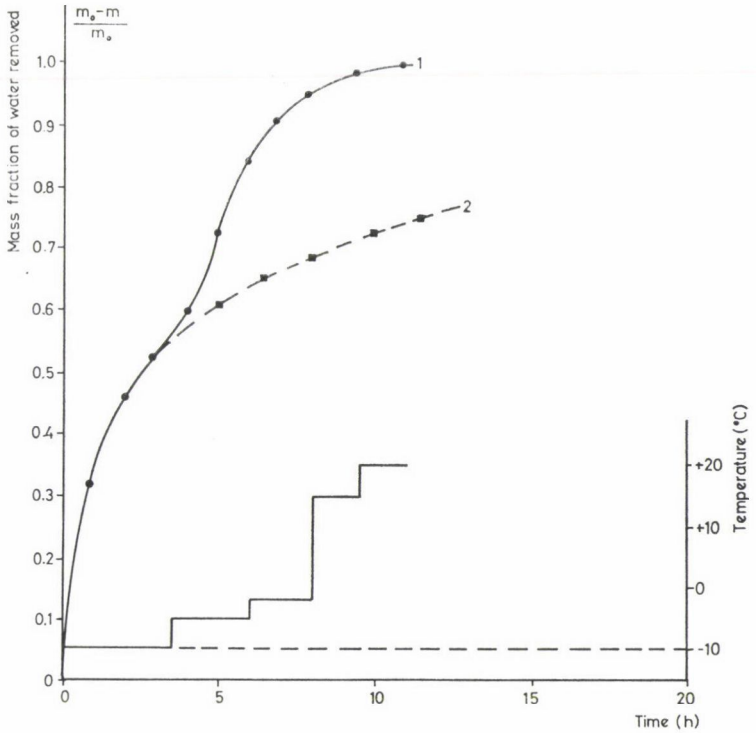

Fig. 6. Carrots (disc thickness = 4 mm); drying by temperature programming (1) and drying at constant temperature (— 10 °C) (2)

3.1. Temperature programming

In order to curtail drying time without sacrificing product quality, a temperature programming technique simulating closely the temperature profile of food samples during freeze drying (BOEH-OCANSEY, 1984) in vacuo, was adopted. A drying regime was operated such that at least 50% of the total water present in the food sample was removed at temperatures around —10 °C; about 70 to 80% of total water was removed at —5 °C; 90% total moisture was removed around 0 °C, and the remaining 10% removed at temperatures greater than 0 °C but never exceeding 20 °C. The programming of temperature was conducted in a stepwise gradual fashion that avoided severe and sharp increments. Temperature changes were made especially when the drying rates tended to fall (Figs. 6 and 7).

Conditions for atmospheric freeze-drying are satisfied by this drying technique as long as the fluidized-bed temperature maintained a temperature in the food sample below its melting point, while the partial vapour pressure of water in the air drier stayed permanently below the triple point value. Under

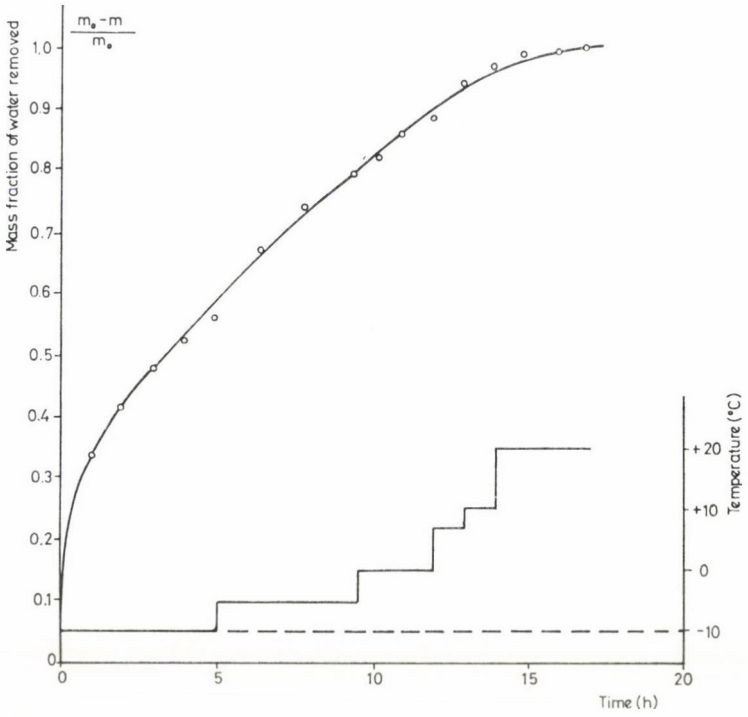

Fig. 7. Drying whole shrimps by temperature programming technique. Maximum diameter (d_{max}) = 10 mm

these circumstances, it would appear that freeze-drying conditions regulated the removal of at least up to 50% of the total water present in the food sample. The rest of the drying regime nonetheless did not impose thermal stresses on the product to induce excessive biochemical degradation. Using the temperature programming technique, whole shrimps (0.20 kg) were dried in approximately 17 h (Fig. 7) while the 4 mm-thick carrot discs (sample *B*, 0.20 kg) required a drying period of only 11 hours (Fig. 6).

3.2. Quality of freeze-air-dried food samples

Assessing the quality of partially freeze-dried and subsequently air-dried, compressed carrot bars, SCHADLE and co-workers (1983) determined among other quality indices, the carotene retention of their samples; a sensory evaluation of the overall acceptability of the products was also undertaken. At a 95% confidence level, little difference was noted between fresh samples, completely freeze-dried and compressed samples and partially freeze-dried then air-dried and compressed samples. Similarly, a sensory panel evaluated carrot samples

dried by the temperature programming technique described above to be of acceptable quality (BOEH-OCANSEY, 1984); the differences noted between thawed, vacuum freeze-dried and atmospheric freeze-air-dried samples were not statistically significant at the 95% confidence level. β-carotene retention for 50% vacuum freeze-dried then air-dried carrots was 76% when compared to fresh samples, however, when 90% of total moisture was removed by vacuum freeze drying and thereafter the remaining 10% was removed by air drying, β-carotene retention was reported by SCHADLE and co-workers (1983) to be 95%. The low-temperature fluidized-bed atmospheric technique with temperature programming resulted in β-carotene retention of 98% when carrot samples were similarly dried. Colour degradation in the shrimp samples was 73% when astacene content of the fluidized-bed dried product was compared to that of frozen analogues. This result nonetheless represented a good performance of the novel drying technique as colour deterioration recorded for vacuum freeze-dried samples of the same batch was in the neighbourhood of 70%.

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CLASSIFICATION OF PAPRIKA QUALITY USING PATTERN RECOGNITION METHODS BASED ON ELEMENTAL COMPOSITION

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Twenty-three paprika samples of different quality, according to the definition of the HUNGARIAN STANDARD (1983) grown in the same year and at the same geographical location, were analysed on the basis of their ash contents and the elemental composition of the ash. The aim of the examinations was to determine whether the samples could be classified according to quality on the basis of the characters tested. The Fe, Ca, K and Mg contents of the ash samples prepared from paprika plants were determined with a Zeiss AAS 1 atomic absorption spectrometer, the P content spectrophotometrically, and the Si, Pb, Mn, Al, Ni, Cu, B and Ti contents by means of optical emission spectral analysis. After processing the data with an EMG-666 table-top computer using the Karhunen-Loeve transformation, the Fisher weight and the K-nearest neighbour pattern recognition methods, it was found that the samples could be efficiently classified according to the paprika species.

Keywords: pattern recognition methods, paprika quality, atomic absorption spectrometer

The rapid spread of modern, high-capacity analytical methods and the enormous development in computer techniques have opened up new paths in the field of food quality control, too. The rapid development of quality control methods has been promoted to a great extent by the use of pattern recognition methods in analytical chemical data processing. Over the last ten years more and more researchers have used pattern recognition to process food analytical data. The literature is particularly rich in papers on the classification of alcoholic drinks, wines and whisky brands. KWAN and KOWALSKI (1978, 1980a, b) reported on the data processing of sensory evaluations. SAXBERG and co-workers (1978) and KWAN and KOWALSKI (1980c) presented the results of gas chromatography determinations, while KWAN and co-workers (1979) classified wines on the basis of their elemental composition, and BORSZÉKI and co-workers (1983) according to growing area.

KARIMIAN-TEHERANI and co-workers (1983a, b) examined the trace element content of paprika samples in various parts of the plant (leaf, stem, roots, fruit with 20% seeds and without seeds) between July and October. These

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authors used neutron activation analysis to determine the concentration of Zn, As, Cr, Mn, K and Br, and found that the quantity of K, Mn and Br was significantly higher in seedless samples than in fruit samples containing 20% seeds.

The aim of the present experiments was to determine whether the sweet, semi-sweet and hot blends of paprika spice ground from paprika fruits grown in the Kalocsa region in 1982 could be classified on the basis of the ash content and dry matter content of the samples and from the data on elemental composition.

1. Materials and methods

1.1. Determinations by atomic absorption spectrometer

The Fe, Ca, K and Mg contents of paprika ash samples obtained from the Somogy county Institute for Food Testing and Chemical Analysis were determined in an AAS-1 (Carl Zeiss, Jena) flame atomic absorption spectrometer, and the P content using the ammonium phosphomolybdate spectrophotometric method (KOCH, 1972).

1.2. Determinations by optical emission spectral analysis

Trace and minor elements were determined by optical emission spectral analysis using the total evaporation method, on a PGS-2 (Carl Zeiss, Jena) plane-grating spectrograph.

Working conditions:

Blaze wavelength: 280 nm
Reciprocal linear dispersion: 0.76 nm/mm
Slit width: 0.020 mm
Three-step filter: 100/50/10%
Emulsion: Agfa-Gevaert Scientia 23D56
Source: d.c. arc, 12 A
Sample quantity: 30 mg
Total evaporation: 150 s

When preparing the samples 100.0 mg ash samples were homogenized with 100.0 mg spectrally pure graphite powder in an agate mortar, after which 30.0 mg of the mixture were filled into the bore of a cup electrode. In addition to the elements determined by atomic absorption and spectrophotometry, Si, Pb, Mn, Al, Ni, Cu, Ag, Na, B, Ti and Sr could also be detected. The concentrations of Si, Mn, Ti, Cu, Sr and Ni, which were present in analytical quan-

tities in all the samples, were measured as the blackening value proportional to the logarithm of the concentration. The analytical lines were as follows:

Si	251.60 nm	Cu	324.75 nm
Mn	293.30 nm	Sr	338.07 nm
Ti	308.80 nm	Ni	338.06 nm

1.3. Data processing

The processing of the analytical data was carried out using pattern recognition methods. For a brief summary of these methods, let denote

$$x_{ij} \qquad i = 1, 2, \ldots, n; \qquad j = 1, 2, \ldots, m$$

the jth feature of the ith sample, and

$$c_{kl} = \frac{1}{n} \sum_{i=1}^{n} (x_{ik} - \bar{x}_{.k})(x_{il} - \bar{x}_{.l}) \quad k, l = 1, 2, \ldots, m$$

the empirical covariances, where

$$\bar{x}_{.k} = \frac{1}{n} \sum_{i=1}^{n} x_{ik} \quad k = 1, 2, \ldots, m$$

are the sample means of the kth feature. In our case the number of samples *(n)* is 23, and we have 11 features *(m)*.

Karhunen-Loeve (K—L) transformation is used for reducing of dimensions and displaying the sample points. Let

$$\lambda_1 \geqslant \lambda_2 \geqslant \ldots \geqslant \lambda_m$$

denote the eigenvalues of the $C = \left[c_{kl}\right] \begin{matrix} l = 1, 2, \ldots, m \\ k = 1, 2, \ldots, m \end{matrix}$ covariance matrix, and

$$\underline{V}_k = \begin{bmatrix} V_{k1} \\ V_{k2} \\ \vdots \\ V_{km} \end{bmatrix} \quad k = 1, 2, \ldots, m$$

the corresponding orthonormed eigenvectors. The principal components of the ith sample are

$$K_{ik} = \sum_{j=1}^{m} (x_{ij} - \bar{x}_{.j}) V_{kj} \quad k = 1, 2, \ldots, m$$

Having the first $m' < m$ principal components,

$$\mathrm{var}_{m'} = \frac{\sum_{k=1}^{m'} \lambda_k}{\sum_{k=1}^{m} \lambda_k} 100\ \%$$

is the information content left in the transformed data.

Autoscaling is used for normalizing. The normalized features of the ith sample are

$$x_{ij}^* = \frac{x_{ij} - \bar{x}_{.j}}{\sqrt{\sigma_j}} \quad i = 1, 2, \ldots, n; \quad j = 1, 2, \ldots, m$$

Fisher weights are used for selecting the most important features. This weight for the kth feature with respect to classes A and B is

$$F_{AB}^k = \frac{(\bar{x}_{Ak} - \bar{x}_{Bk})^2}{v_{Ak} + v_{Bk}} \quad k = 1, 2, \ldots, m$$

where $\bar{x}_{Ak}$, $\bar{x}_{Bk}$, v_{Ak} and v_{Bk} are the sample means and variances for the kth feature in classes A and B.

K-nearest neighbour method is used as a decision making rule. A sample point is classified into class A if among its K nearest neighbours from the learning set, the most learning points belong to class A. Selecting the nearest neighbours, the Euclidean distance of two samples with features

$$x_1, x_2, \ldots, x_m \quad \text{and} \quad y_1, y_2, \ldots, y_m,$$

is used, namely:

$$\left[\sum_{i=1}^{m} (x_{ik} - y_{il})^2\right]^{\frac{1}{2}}$$

A more detailed description of these methods is given in BORSZÉKI and co-workers (1983) and VARMUZA (1980).

2. Data processing results

When processing the analytical data (Table 1) an attempt was made to carry out a classification according to quality on the basis of the ash content, the Fe, Ca, K, Mg and P contents of the samples, and the data obtained from optical emission spectral analysis (Si, Mn, Ti, Cu, Sr and Ni). The two-dimensional representation of the twelve-dimensional data matrix involved a significant loss of information due to the K—L transformation ($\mathrm{var}_2 = 58\ \%$; Fig. 1).

With help of the Fisher weight equation the parameters which could be considered from the point of view of classification were ranked in order of decreasing weight (Table 2).

Table 1

Data used in the calculations

Sample number	Micro element content[a]						Ash	Ca	K	Mg	P
	Si	Mn	Ti	Cu	Ni	Sr	(%)	in dry matter (g kg^{-1})			
774	141	21.8	88.2	112	27.4	58.2	6.21	1.34	40.3	2.59	3.97
632	144	20.3	104	116	33	43.5	6.33	1.77	36.9	3.00	4.01
672	129	18.5	41.5	134	16.5	43.3	6.07	1.80	37.2	2.43	3.67
705	133	17.1	41	115	26.3	64	6.19	1.73	34.5	2.28	4.03
543	144	17.8	70.7	121	28.3	57.8	6.34	1.91	38.4	2.95	4.09
5699	137	17.1	86	108	23.8	56	6.5	1.82	44.1	2.5	3.95
669	138	25.4	73	136	22.5	58	6.13	1.9	35.3	2.99	3.73
687	160	30.7	106	108	37	56	6.23	1.94	38.36	2.49	4.13
824	146	27.5	90.5	111	23	49	6.72	1.73	30.7	2.33	3.48
876	156	16.8	91.6	91.9	40.9	48.9	6.98	1.34	40.6	2.27	3.69
564	146	9.73	45.9	98	12.7	38.3	7.26	2.35	41.6	2.27	3.75
746	143	17.2	69	110	29.8	62.8	7.13	2.25	40.8	2.63	3.99
690	143	11.1	81.2	103	19.2	54.1	7.39	1.93	46.22	2.77	3.63
873—874	142	14.2	100	101	38.1	53.6	6.86	1.46	38.95	2.15	3.67
804	157	11.1	87	104	31.3	66.8	7.06	1.74	41.93	1.98	3.76
4945	160	16.3	92.7	100	36.1	58.7	7.55	2.24	46.05	2.05	3.57
626	139	17.4	105	103	37	67.1	7.32	2.16	47.1	2.54	3.44
825	161.5	12.6	105	96	53	70.9	7.28	2.15	41.3	2.49	3.66
623	162	28.9	118	125	83.2	68.3	6.89	2.79	40.9	2.61	2.75
742	167	14.8	126	97.6	59.7	55.3	6.57	2.23	39.8	2.57	3.15
885	160	15.4	116	116	44.6	55.5	7.17	2.28	39	2.24	3.78
733	164	12.4	142	79.3	24	53.8	8.5	2.98	49.5	2.10	3.52
915	158	13.6	108	71.7	35	55.9	7.01	1.89	39.2	2.22	3.76

[a] The data measured by optical emission spectral analysis are hundredfold logarithm intensity values originating from measurements of the blackening of the analytical lines

Table 2

Micro element contents in the ash of different paprikas

Sweet — hot		Sweet — semisweet		Semisweet — hot	
Parameter	F.w.	Parameter	F.w.	Parameter	F.w.
ash content	3.48	ash content	3.56	Ca	0.82
Si	2.64	Cu	1.98	Ni	0.65
Ca	1.50	Si	1.36	Si	0.45
P	1.20	Mg	0.73	Ti	0.41
Ni	1.11	P	0.50	Sr	0.35
Ti	0.76	Mn	0.14	P	0.34
Mg	0.76	Ti	0.08	ash content	0.28
Sr	0.49	Ni	0.08	Cu	0.18
Mn	0.39	Ca	0.02	Mg	0.008
Cu	0.33	Sr	0.001	Mn	0.007

F.w. = Fisher weight

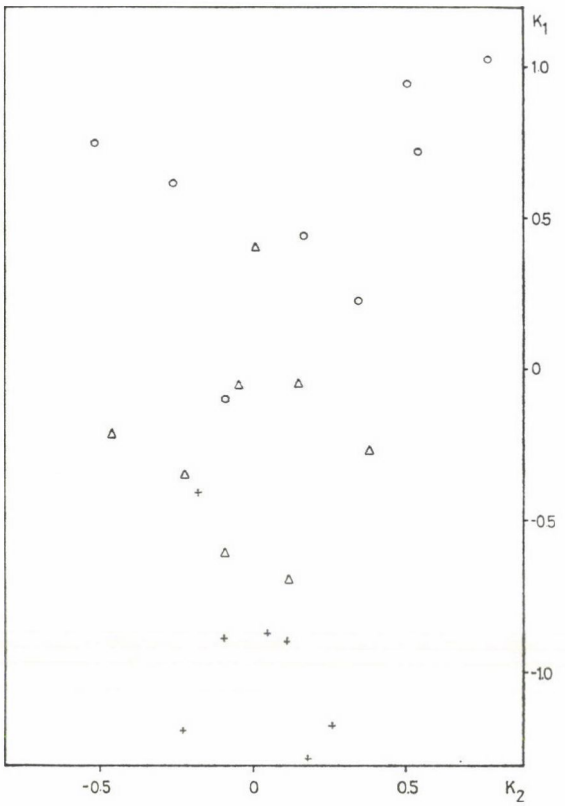

Fig. 1. Two-dimensional representation of hot, sweet and semi-sweet samples on the basis of ash, Fe, Ca, K, Mg, P, Si, Mn, Ti, Cu, Sr and Ni contents. There is 58.0% information transfer. The data were normalized using the autoscaling method. K_1 and K_2 are new variables formed from the eigenvectors associated with the two highest eigenvalues of the covariance matrix of the data matrix. Signs: ○ hot, + sweet, △ semi-sweet

In further processing the data matrix the Mn and Mg data were no longer considered, and the new K—L transformation led to an efficient classification according to quality. There was a slight decrease in the loss of information arising in the course of transformation ($var_2 = 60.2\%$; Fig. 2).

3. Conclusions

Ash samples of three qualities of paprika (sweet, semi-sweet and hot) grown on the same site in the same year were analysed to discover whether classification according to quality was possible on the basis of the ash content, minor and trace element contents of the samples. The data determined by various analytical techniques were processed using several pattern recognition

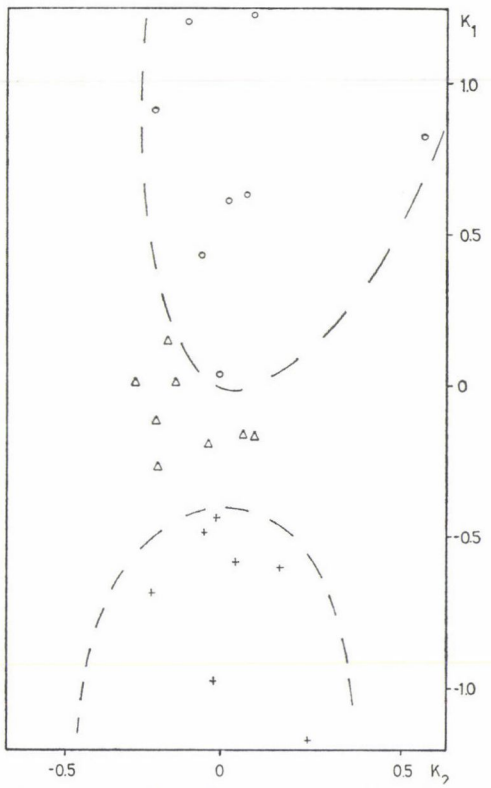

Fig. 2. Two-dimensional representation of hot, sweet and semi-sweet samples on the basis of ash, Fe, Ca, K, P, Si, Ti, Cu, Sr and Ni contents. There is 60.2% information transfer. The data were normalized using the autoscaling method. K_1 and K_2 are new variables formed from the eigenvectors associated with the two highest eigenvalues of the covariance matrix of the data matrix. Signs: ○ hot, + sweet, △ semi-sweet

methods. The calculations showed that, after selecting the most suitable features, paprika samples could be efficiently classified according to quality on the basis of these parameters. This could be of importance for quality control. In order to check the reliability of the rankings calculations were made in which the individual sample points were assumed to be unknown, in which case the procedure gave the classification for the $K = 4$ or $K = 5$ nearest neighbour. The K-nearest neighbour method was applied both for the two greatest main components (two-dimensional K—L transformation) and for the three greatest main components (three-dimensional K—L transformation) (Table 3).

The significance of the ranking shows as a percentage the number of tested samples which were correctly placed by the pattern recognition method in the relevant class.

It can be seen from the results of the measurements and calculations that paprika mixtures of various qualities prepared from paprikas grown on the

Table 3

Reliability of the ranking calculations

	Reliability of ranking (%)		
	Sweet	Semi-sweet	Hot
Two-dimensional K-L transformation			
$var_2 = 60.2\%$			
$K = 4$	100	88.8	77.8
$K = 5$	71.4	66.6	77.8
Three-dimensional K-L transformation			
$var_3 = 87.0\%$			
$K = 4$	100	77	62.5
$K = 5$	100	77	75

K = nearest neighbour number

same site in the same year can be distinguished from each other according to variety on the basis of their elemental composition and ash content. The method outlined here could thus contribute to the elaboration of a quality control technique.

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FRACTIONATION AND CHARACTERIZATION OF *PISUM SATIVUM* ALBUMINS

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Study of the effect of pH on the solubility of the albumins extracted from Lincoln seeds showed that the minimum solubility of albumins was at pH 4.1.

The total pea albumins were fractionated into at least six components by ion exchange chromatography on DEAE-cellulose column as well as gel filtration chromatography on Sephacryl S-200 column.

The spectrophotometric analyses for the albumin components separated by gel filtration indicated the contamination of components *A* and *D* with nucleic acids. No protein was detected in peak *E* when subjected to polyacrylamide gel electrophoresis or by reaction with Folin reagents.

Examination of total albumins by polyacrylamide gel electrophoresis revealed two major and more minor bands after staining with Amido Black.

The amino acid analyses showed that the total albumins and their components contained relatively high levels of glutamic and aspartic acid as well as leucine+isoleucine. Substantial amounts of lysine and arginine were also recorded in the albumins. The level of cystine was low.

Keywords: pea, proteins, albumins

The growing need of food and feed protein stimulate the research work relating to legume seeds. Although the globulins are the major storage proteins of legumes, the albumin fraction contributes a quantitatively important proportion of the seed protein of *Pisum sativum*. Nevertheless the albumin fraction was not yet thoroughly investigated. After earlier work of Tkachenko (1970) and Gofman (1972), gel filtration chromatography on Sephadex G-100 and G-200 was used for fractionating and determining the molecular size of the albumin component of *Pisum sativum* (Grant et al., 1976; Jakubek & Przybylska, 1979). Recently a systematic work was carried out by Schroeder (1982, 1984).

The work reported here deals with fractionation and characterization of *Pisum sativum* albumins including the molecular size, the amino acid composition and some other properties.

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1. Materials and methods

1.1. Preparation of dry defatted meal

The dehulled *Pisum sativum* (Lincoln) seeds were ground in a coffee grinder to a fine powder, then blended with ice cold acetone. The defatted material was air-dried overnight at room temperature and stored in a closed container at 4 °C until required.

1.2. Extraction of pea albumins

The fat free meal was extracted with distilled water, using a ratio of 1:20 (w per v) for meal to water. The suspension was stirred for 2 h at 4 °C, filtered through muslin and then centrifuged at 5000 r.p.m. for 30 minutes. The residue was extracted once more by the same procedure and the supernatants were combined, dialyzed against distilled water for 72 h at 4 °C. The dialyzed extract was centrifuged at 10 000 r.p.m. for 30 min and the clear supernatant freeze-dried.

1.3. Effect of pH on the solubility of pea albumins

Protein solutions (1 mg cm^{-3}) were prepared by dissolving the freeze-dried protein in distilled water. Aliquots of 15 cm^3 were adjusted to the desired pH by adding either 5 mol cm^{-3} HCl or NaOH and then stirred for 15 min at room temperature. Turbidity of protein solutions at various pH values was read at 600 nm. After leaving overnight at 4 °C, the protein solutions were centrifuged at 10 000 r.p.m. for 30 min. Protein concentration in the supernatant was measured by recording the absorbance at 280 nm.

1.4. Fractionation of pea albumins on DEAE-cellulose column

A preswollen microgranular form of DEAE-cellulose (DE 52) was equilibrated according to the method recommended by the manufacturer's instructions. The required amount of exchanger was equilibrated with 20 mmol dm^{-3} phosphate buffer, pH 7.1. The protein solution in the starting buffer was applied on the column via a three way tap and eluted from the column first with equilibrating buffer and then with the starting buffer containing increased NaCl concentration (stepwise elution).

Fractions of 10 cm^3 were collected at a flow rate of 0.5 cm min^{-1}. The absorbance at 260 and 280 nm was measured for each fraction.

1.5. Gel filtration chromatography

Sephacryl S-200 equilibrated in 0.1 mol dm^{-3} Tris-HCl(pH=7.6) containing 0.5 mol dm^{-3} NaCl was packed into a column (2.5×100 cm). The protein sample was then loaded on the column from a suitable reservoir via a three way tap. The height of the buffer reservoir was then adjusted to give a flow rate of about 20 cm^3 h^{-1} and fractions of a suitable size were collected using the LKB Vectorac 7000 Fraction Collector (LKB product, Bromma I, Stockholm, Sweden).

The optical density of each fraction was monitored at 280 nm. A portion of each fraction was analyzed by phenol-Lowry method at 750 nm (LOWRY et al., 1951).

1.6. Polyacrylamide gel electrophoresis

Electrophoresis of protein fractions for a routine check on enzyme purity was performed on 7.5% (w/v) polyacrylamide gels. The method used was a modification of the disc electrophoresis as described by DAVIS (1964). The gels were examined for protein bands by spectrophotometric scanning or by the use of protein specific stains, with 1% Amido Black or 0.2% Coomassie Brilliant Blue.

The excess of stain was removed by repeated washing in 7% (v per v) aqueous acetic acid when Amido Black was used (NAGAI et al., 1964) or glacial acetic acid : ethanol : water (2:5:13 by volume) as destaining solution for Coomassie Brilliant Blue until the bands were distinctly dark blue or deep blue, respectively, in a colourless background. The gels stained by Amido Black could be scanned at 600 nm and at 609 nm for those stained by Coomassie Brilliant Blue on the Gilford 2400-S spectrophotometer, fitted with the 2410-S linear transport attachment, to record the positions of the protein bands.

1.7. Determination of amino acids for protein fractions

Appropriate amount of the total pea seed albumins and the purified albumin obtained by fractionation on Sephacryl S-200 column, were hydrolyzed in sealed tubes at 110 °C for 24 h with 6 mol dm^{-3} HCl. The excess HCl was then evaporated under vacuum, with occasional addition of distilled water for 3–5 times. The residue was dissolved in 10% isopropanol (v/v), then filtered, and the final volume was made up to 5 cm^3 in a measuring flask with the same solvent. The percentage of each amino acid was determined by paper chromatography and spectrophotometrically using three types of systems and ninhydrin–cadmium acetate in acetone as a reagent solution (HEILMANN et al., 1957).

2. Results and discussion

2.1. Effect of pH on the solubility

Pisum sativum albumins showed a characteristic solubility curve with changing pH values (Fig. 1). The solubility curve of pea albumins over pH ranged from 2–12 indicated that the solubility of protein at alkaline and acidic pH values was greater than that observed at neutral pH and there was a point of minimum solubility at pH 4.1 (Fig. 1). The minimum solubility of the pea proteins occurred at pH 4.1 and could be attributed to the proximity of this pH value to their isoelectric points.

Solubility of several legume proteins as a function of pH, similar to Fig. 1 have been reported (FAN & SOSULSKI, 1974; VOSE, 1980; EL MORSI, 1982).

2.2. Fractionation of albumins by ion exchange chromatography

The elution profile of DEAE-cellulose column is shown on the diagramme in Fig. 2. It is clear that the pea albumins were separated into at least six components marked *a*—*f* in the order of their elution from the column. The components *a*, *b* and *c* were eluted with the starting buffer.

On the other hand, components *e* and *f* were strongly bound and eluted with buffer containing 0.2 mol sodium chloride, respectively (Fig. 2). The fourth

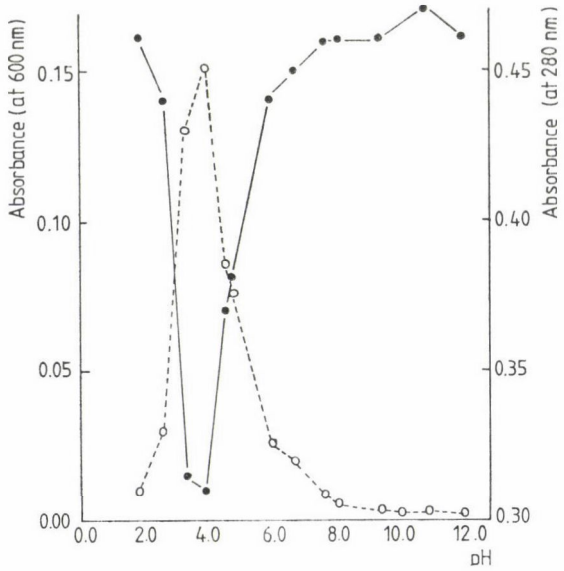

Fig. 1. Effect of pH on the solubility of pea albumins. o— — — —o: 600 nm; ●————●: 280 nm

Table 1

Ultraviolet-visible absorption characteristics and V_e per V_0 of the albumin components obtained by gel filtration chromatography

Component	$E_{max.}$ (nm)	$E_{min.}$ (nm)	A_{280} per A_{260}	A_{750} (nm)	V_e per V_0
a	260	244	0.64	2.99	0.91
b	275	250	1.11	1.70	1.15
c	277	252	1.30	2.32	1.5
d	256	248	0.70	0.238	2.06
e	253	240	0.57	0.00	2.19
f	273	240	1.03	0.076	2.37

Symbols: E: extinction; A: absorption; V_e per V_0: relative elution volume

component emerging from the column (peak *d)* was weakly bound to DEAE-cellulose and was eluted with 0.1 mol sodium chloride in 20 mmol dm^{-3} phosphate buffer, pH 7.1. The major albumin component eluted from the DEAE-cellulose column was the protein emerging with 0.2 mol sodium chloride in the starting buffer.

The ratio of absorbance at 280 nm to that at 260 nm (A_{280}/A_{260}) was estimated for each albumin component, eluted from DEAE cellulose column. The values obtained were: 1.16 *(a)*, 0.95 *(b)*, 1.3 *(c)*, 1.2 *(d)*, 1.4 *(e)* and 1.2 *(f)*, respectively (Table 1). These results indicated varying contamination with nucleic acids (WARBURG & CHRISTIAN, 1942). The lowest A_{280} / A_{260}

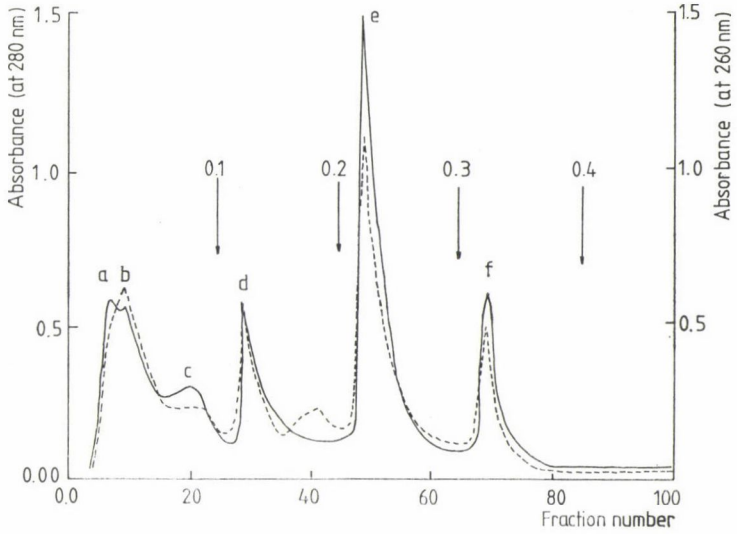

Fig. 2. Chromatography of total pea albumins on DEAE-cellulose column. ———: A_{280}; — — — —: A_{260}. The molarity of buffer was 0.1, 0.2, 0.3 and 0.4, respectively. *a—f*: signs of the dissolved components

ratio for component *b* pointed to the presence of a high level of nucleic acid. On the other hand, the ratio of A_{280}/A_{260} for component *e* (1.4) suggested very low contamination with nucleic acids.

2.3. Fractionation of pea albumins by gel filtration chromatography

Pea albumins (200 mg), extracted from the seeds of *Pisum sativum* variety Lincoln, as described in para. 1 were dissolved. After centrifugation to remove any undissolved material, the clear protein solution (6 cm^3) was loaded on to the top of a Sephacryl S-200 column. The column was eluted with the same buffer and fractions of 6 cm^3 were collected. The absorbance at 280 nm was recorded for each fraction. A portion of fractions with highest absorbance at 280 nm in each peak, were analyzed for proteins by the method of Lowry at 750 nm (LOWRY et al., 1951). Absorbance readings were plotted against tube number to give the elution profile shown in Fig. 3.

It is clear that pea seed albumins consist of at least six components designated *A*—*F* in the order of their elution from the column (Fig. 3). Three of the peaks obtained were high (*A*, *C* and *F*) while the others were low (*B*, *D* and *E*). The results of protein determination at 750 nm by the Lowry method indicated that components *A*, *B* and *C* contain larger amounts of protein than components *D* and *F*. No proteins could be detected in peak *E* when subjected to polyacrylamide gel electrophoresis or by reaction with Folin reagent.

The Sephacryl S-200 column was calibrated with five proteins of known molecular weights (lactate dehydrogenase, bovine serum albumin, ovalbumin,

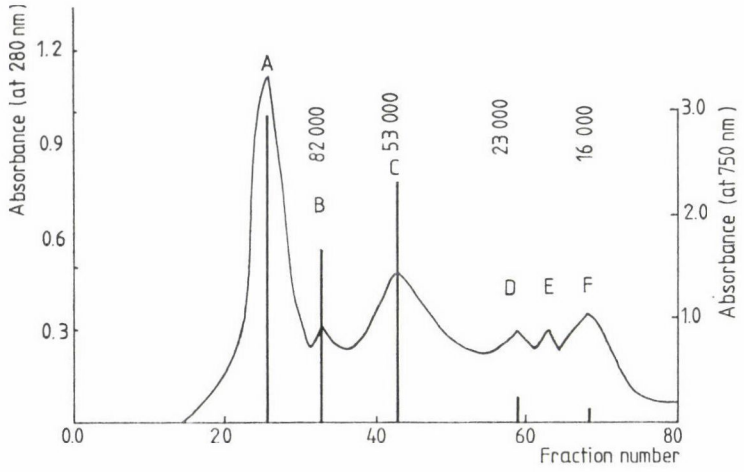

Fig. 3. Chromatography of total pea albumins on Sephacryl S-200 column showing the fractions of molecular mass. ———: A_{280}; ———: A_{750}. *A*—*F*: signs of the eluted components

myoglobin and lysozyme). This column was used for the molecular weight determination of the pea albumin components according to the method described by ANDREWS (1965). The albumin components were resolved by gelfiltration according to their molecular size. The elution curve obtained (Fig. 3) showed that component *A* (peak A) eluted through the void volume of the Sephacryl S-200 column indicated the presence of protein of high molecular weight ($>$ 200 000). This large size protein might be one of the *Pisum sativum* albumin components or resulted from the association of smaller protein molecules. Similar association reactions have been reported for soybean protein (WOLF & BRIGGS, 1958).

The other albumin components with molecular weights of approximately 82 000, 53 000, 23 000 and 16 000 (Fig. 3) were separated during this investigation. The results of molecular weight determination (Fig. 3) indicated the similarity between some of the albumin components obtained in this work with those reported in the literature (GRANT et al., 1976; JAKUBEK & PRZYBYLSKA, 1979).

Ultraviolet absorption spectra of peaks eluted from Sephacryl S-200 column are presented in Fig. 4. Peak A with maximum absorbance at 260 nm indicated that this protein is nucleoprotein or protein contaminated with nucleic acids (OBARA & KIMURA, 1967; MORITA & YOSHIDA, 1968). It is known that pure nucleic acids are absorbed at 260–265 nm, and the peak with lowest A_{280}/A_{260} ratio appeared to contain highest levels of nucleic acids (WARBURG & CHRISTIAN, 1942). Therefore peak E with its lowest A_{280}/A_{260} ratio, appeared to contain the highest level of nucleic acids (Table 1), and its ultraviolet absorption spectrum (Fig. 4) support our conclusion. A similar finding was re-

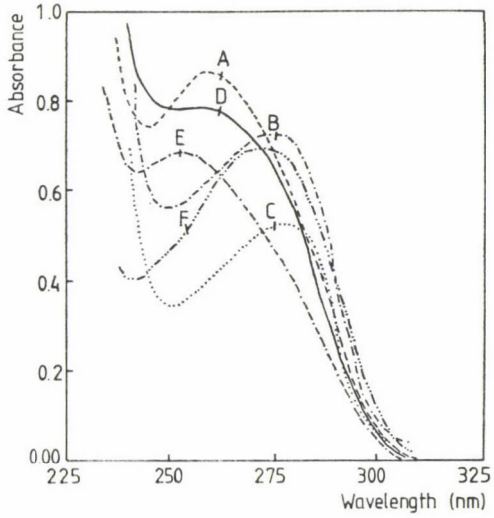

Fig. 4. Ultraviolet absorption spectra of pea albumin components (A—F) obtained from Sephacryl S-200 column

ported by TKACHENKO (1970) who found some pea proteins eluted from DEAE-cellulose column contaminated with nucleic acids, also a non-protein peak emerged during his fractionation.

The ultraviolet absorption spectra of components *B* and *C* were characterized by maximum absorption at 275 and 277 nm and minimum absorption at 250 and 252 nm, respectively (Fig. 4). The ratios of A_{280}/A_{260} for these two components (Table 1) suggested very little contamination with nucleic acids (WARBURG & CHRISTIAN, 1942).

2.4. Polyacrylamide gel electrophoresis

Examination of the total pea seed albumins by polyacrylamide gel electrophoresis revealed two major and more minor bands after staining with Amido Black. The densitometric scanning of polyacrylamide gel electrophoretic patterns of the total albumin is presented in Fig. 5. The relative mobility (R_m) values of the bands were, 0.48, 0.61, 0.66, 0.76, 0.81, 0.88 and 0.97, respectively, and the band with R_m value of 0.61 was the major albumin component.

2.5. Amino acid composition of pea albumins

The fractions of each albumin component eluted from Sephacryl S-200 column (Fig. 3) were pooled, dialyzed against distilled water and lyophilized. The results of amino acid analyses of some of the albumin components and total albumins are shown in Table 2. The amino acid composition of the components showed similar character but some differences were also observed.

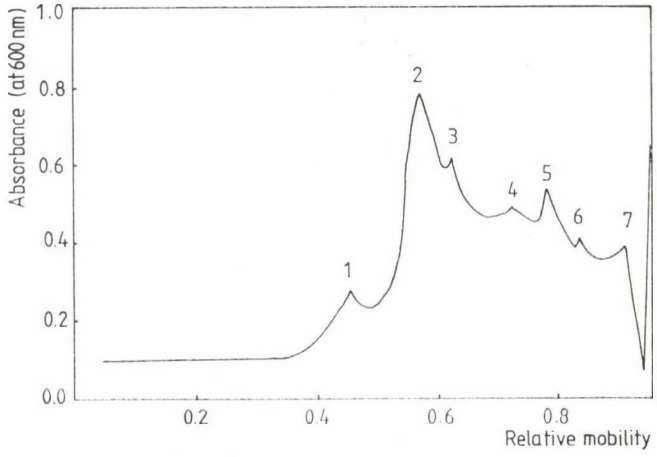

Fig. 5. Densitometric scanning of polyacrylamide gel electrophoretic patterns of pea albumins. The relative mobility (R_m) of components are as follows: 1 = 0.48; 2 = 0.61; 3 = 0.66; 4 = 0.76; 5 = 0.81; 6 = 0.88; 7 = 0.97

Table 2

Amino acid composition of pea albumins
(g amino acid per 100 g protein)

Amino acid	Total albumins	Albumin components obtained by gel filtration			
		A	*B*	*C*	*F*
Alanine	5.57	6.91	6.69	5.14	4.34
Arginine	7.60	13.21	10.51	8.64	3.77
Aspartic acid	13.20	12.34	13.42	12.43	15.13
Cystine	0.51	0.66	0.53	0.62	1.40
Glutamic acid	14.66	12.16	17.63	14.73	17.23
Glycine	4.90	3.00	5.70	4.20	2.90
Histidine	2.12	0.54	2.10	3.15	2.67
Isoleucine, Leucine	13.30	13.32	16.30	13.40	17.22
Lysine	8.11	10.73	9.12	8.87	9.23
Methionine	2.27	0.70	1.66	2.56	2.28
Phenylalanine	5.20	4.56	0.00	5.24	4.99
Proline	Trace	Trace	Trace	Trace	Trace
Serine	4.34	3.40	2.67	3.45	2.23
Threonine	4.61	5.70	5.79	4.68	3.89
Tryptophan	0.90	N.T.	N.T.	N.T.	N.T.
Tyrosine	8.11	7.33	8.00	8.98	7.76
Valine	2.98	2.87	3.34	2.35	3.12

N.T.: Not determined

The major amino acids of the total albumins and their components were glutamic and aspartic acids as well as leucine+isoleucine (Table 2). In addition to these amino acids, components *A*, *B* and *C* contained higher concentration of arginine and substantial amounts of lysine.

All components contained relatively low levels of cystine and some of them also low level of methionine. It has been suggested by JOHNSON and LAY (1974) that protein components richer in sulphur containing amino acids may be used to enhance the level of methionine and cystine in edible seed legumes, but BOULTER and co-workers (1978) did not support this view.

The albumin components *B*, *C* and *F* contained more methionine, the first and sometimes the second limiting amino acid in legumes (BHATTY, 1982).

The amino acid compositions reported in this study were in some aspects similar to those reported by other investigators (BHATTY, 1982; BOULTER & DERBYSHIRE, 1971). Neither tyrosine nor phenylalanine appeared in significant quantity in component *B*, separated on a Sephacryl S-200 column.

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XYLOSE ISOMERASE ACTIVITY OF YEASTS AND YEAST-LIKE ORGANISMS

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Isomerization between certain aldo- and keto-sugars due to presence of D-xylose isomerase in some yeasts and yeast-like organisms is described. Among 465 representatives of these organisms almost 20% of the strains tested gave positive reaction on a synthetic selection medium with D-xylose, revealing presence of the enzyme. Considering the price of D-xylose as inducer for xylose isomerase, selection of the strains of xylanolytic activity was carried out. For this purpose selection media consisting of gels prepared from crosslinked derivatives of xylan and synthetic Yeast Nitrogen Base were used. Only 9 strains among the xylose isomerase producers were proved to be simultaneously endo-acting xylanase producers.

Considering the morphology, xylose isomerase and xylanase producing ability of the organisms, *Wingea robertsii* was found to be the most suitable xylose isomerase producer. The extent of the isomerase reaction was evaluated radiometrically. Replacement of D-xylose in the production medium by xylan resulted in increasing xylanolytic activity in the medium, the xylose isomerase activity remained, however, unaltered. The harvested and freeze-dried cells of *Wingea robertsii* were permeabilized and immobilized into the crosslinked polyethylenimine matrix. Conversion of D-glucose to D-fructose owing to the unspecificity of the enzyme concerned was studied with (U-^{14}C) D-glucose as a substrate and with uniformly labelled starch ^{14}C in an enzyme system consisting beside the xylose isomerase of alpha- and gluco-amylase. Concentration of D-fructose in the reaction mixture reached 45% of the total sugars.

Keywords: xylose isomerase, yeasts, yeast activity

D-xylose isomerase (D/+/-xyloseketolisomerase EC 5.3.1.5) is an enzyme predominantly present in microbial cells and catalyzes reversibly isomerization between certain aldoses and ketoses. The physiological role of the enzyme is probably in mediating the formation of D-xylulose from D-xylose. D-xylulose is phosphorylated then to enter the pentose phosphate pathway (MORTLOCK & WOOD, 1964; BISSON & MORTLOCK, 1969; DAVID & WIESMEYER, 1970). However since its unspecificity in the D-glucose isomerization was reported the enzyme has been the subject of intensified research (MARSHALL & KOOI, 1957). The enzyme is used now on industrial scale to manufacture fructose syrups for the food industry and for the preparation of special medical formulae. In addition to its elaboration and industrial application it is at present the third important enzyme preparation, following the proteolytic and amylolytic enzymes (GEYER, 1974; GRAMERA, 1978; HAMILTON et al., 1974). Since its

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detection xylose isomerase activity has been demonstrated in more than 100 different microbial species, mainly bacteria. On the other hand xylose isomerase has so far not been described to occur in fungi. Therefore it is not too surprising that among yeasts the only strains so far reported to produce this enzyme are *Candida utilis* (TOMOYEDA & HORITSU, 1964) and *Rhodotorula gracilis* (HOFER et al., 1971). Aim of this work was to study the ability of yeasts and yeast-like organism to catalyze the aldo-ketose transformation and at the same time to replace expensive D-xylose by cheap sources that is by strains producing xylanase.

1. Materials and methods

1.1. Microorganisms

The strains to be studied in the experiment (i.e. 465) originated from the Czechoslovak Collection of Yeasts and Yeast-like organisms (CCY), Institute of Chemistry, Centrum of Chemical Research, Slovak Academy of Sciences, Bratislava. The following yeasts and yeast-like organisms were used: *Eremascus fertilis, Bullera alba, Ascoidea rubescens, Trigonopsis variabilis, Oospora lactis, Cryptococcus neoformans, C. albidus, C. nigricans, Dioszegia hungarica, Sporobolomyces salmonicolor, Rhodotorula glutinis, Saccharomyces cerevisiae, S. delbrueckii, Ashybya gossypii, Eremothecium ashbyi, Kloeckera corticis, K. javanica, K. apiculata, K. africana, Torulopsis kruisii, Aureobasidium pullulans, Metschnikowia pulcherrima,* mating type a, mating type α, *Saccharomycopsis lipolytica, Candida reukaufii, C. albicans, C. guilliermondii, C. zeylanoides, C. pelliculosa, C. tropicalis, C. pseudotropicalis, C. krusei, C. heveanensis, C. humicola, C. intermedia, C. mesenterica, C. robusta, C. rugosa, C. catenulata, C. monosa, C. curvata, C. muscorum, C. parapsilosis, C. tenuis, C. melinii, C. solani, C. melibiosi, C. japonica, C. arborea, C. brumptii, C. macedoniensis, C. scottii, C. claussenii, C. famata, C. flaveri, C. aaseri, C. corniculata, C. anomala, C. boidinii, C. utilis, C. mycoderma, C. tamarindi, C. polymorpha, C. oregonensis, C. vanriji, C. natalensis, C. langeroni, C. bovina, C. trigonopsoides, C. fimetaria, C. ingens, C. atmosphaerica, C. majoricensis, C. vinaria, C. marina, C. silvicola, C. sorbosa, C. olivarium, C. norvegensis, C. lusitaniae, C. abtusa, C. membranaefaciens, C. aldoi, C. stellatoidea, C. parakrusei, C. mogii, C. maritima, C. shehatea, C. fibrae, C. fragicola, C. citrea, C. rugopelliculosa, C. sorboxylosa, C. iberica, C. steatolytica, C. revautii, C. pseudolipolytica, C. diversea, C. podzolica, Trichosporon pullulans, T. cutaneum, Endomycopsis ovetensis, Trichosporon fermentans, T. capitatum, T. jirovecii, T. brasicae, T. beigelii, T. proteolyticum, T. aculeatum, T. terrestre, T. inkin, T. lutetiae, Brettanomyces bruxellensis, Lipomyces starkeyi, L. lipofer, L. kononenkose, Saccharomycodes ludwigii, Nadsonia fulvescens, Hansenula anomala, H. suaveolens, H. schneggii, H. saturnus, H. subpelliculosa, H. califor-*

nica, H. mrakii, H. glucozyma, H. henricii, H. capsulata, H. minuta, H. holstii, H. canadensis, H. dimennae, H. silvicola, H. beijerinckii, H. bimundalis, H. fabianii, H. petersonii, H. polymorpha, H. nonfermentas, H. wickerhamii, H. jadinii, H. wingei, H. beckii, H. ciferrii, H. muscicola, Hormoascus platypodis, Pichia membranaefaciens, Citeromyces matritensis, Debaryomyces hansenii, D. globosus, Pichia vini, Debaryomyces cantarelii, D. castellii, Pichia fluxuum, Debaryomyces coudertii, Torulospora pretoriensis, Saccharomyces microelipsoides, D. formicarius, Torulospora montana, T. fermentati, T. delbrueckii, Pichia dispora, Debaryomyces formicarius, D. mucosus, Torulospora rosei, D. tamarii, Endomyces magnusii, Saccharomycopsis fibuligera, Saccharomycopsis capsularis, Guillermondiella selenospora, Kluyveromyces polysporus, K. africanus, K. cicerisporus, K. phaffii, K. wikenii, Schizosaccharomyces pombe, Hanseniospora valbyensis, Schwanniomyces occidetalis, Kluyveromyces fragilis, Pachysolen tannophilus, Nematospora coryli, Pichia chambardii, Dekkera bruxellensis, Ambrosiozyma ambrosiae, A. cicatricosa, A. philentoma, A. monospora, Wickerhamia fluorescens, Rhodosporidium toruloides, Leucosporidium scottii, L. capsuligenum, Lodderomyces elongisporus, Wingea robertsii, Filobasidium capsuligenum, Selenotila intenstinalis, Sympodiomyces parvus.

1.2. Selection of the xylanase producing strains

The method used was based on gel prepared in crosslinking reaction from xylan (ex *Fagus silvatica*) applying 2-chloromethyloxirane as a crosslinking agent (Fig. 1). The gel assuming dry weight 5% forming agar-like hydrophylic and transparent medium preserves its substrate properties for the endo-acting xylanase (β-1,4-xylan xylanohydrolase, EC 3.2.1.8) whereas its substrate properties for exo-acting beta-xylosidase are highly suppressed due to crosslinking.

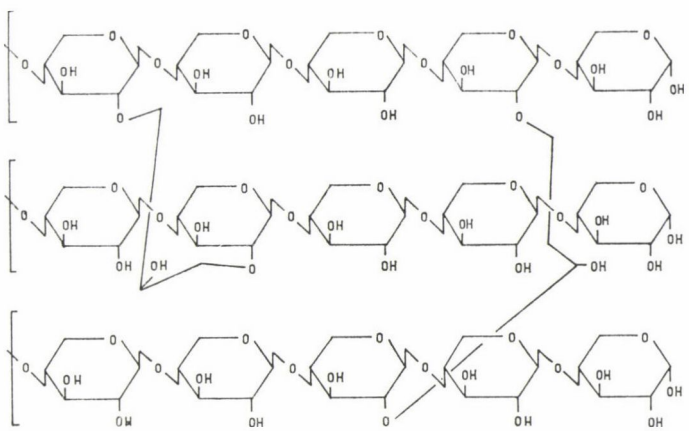

Fig. 1. Structure of the crosslinked xylan

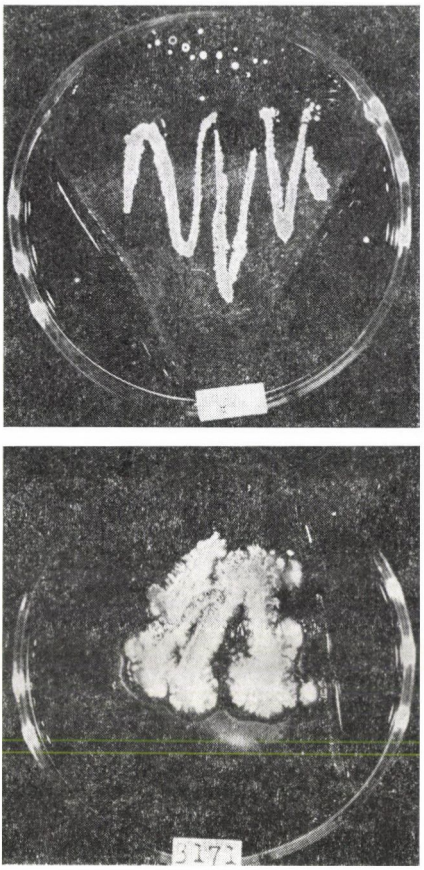

Fig. 2a. The surface growth of *Saccharomyces cerevisiae*; 2b. Production of endo-acting xylanolytic enzymes by *Wingea robertsii* demonstrated through the liquefaction of cross-linked xylan

Moif ication of the polysaccharide by crosslinking was carried out in alkali (ZEMEK & KUNIAK, 1978; ZEMEK et al., 1983). The gel layers prepared were washed free of alkali in tap water and in distilled water. Gel layers 4.5 mm thick were cut into equilateral triangular pieces with side lengths of 15 cm, placed into Petri dishes, immersed into Yeast Nitrogen Base (Oxoid) growth medium (pH 6.0) reaching half the gel thickness and sterilized at 100 °C with streaming steam for 1 h, three times. The β-xylanase producing strains are capable to liquefy the gel (Fig. 2).

1.3. Selection of D-xylose isomerase producers

A synthetic selection medium for yeast-like microorganisms was used ccording to VANDAME and co-workers (1981), containing D-xylose 5.0 g (sterilized separately); NH_4NO_3 2.5 g; Na_2HPO_4 1 g; KH_2PO_4 0.5 g; $CoCl_2 \cdot 6\,H_2O$

0.24 g; $MgSO_4 \cdot 7\,H_2O$ 0.5 g; $MnSO_4 \cdot 5\,H_2O$ 0.5 g; $FeSO_4 \cdot 7\,H_2O$ 0.1 g; EDTA — sodium salt 0.35 g; $CaCl_2$ 0.01 g; $ZnSO_4 \cdot 7\,H_2O$ 0.5 mg; $CuSO_2 \cdot 7\,H_2O$ 0.1 mg; $Na_2MoO_4 \cdot 2\,H_2O$ 0.05 mg; agar 20 g and distilled water to 1 dm^3 (pH adjusted to 7.0).

Positive strains and stock cultures have been maintained on Yeast Malt Agar slants containing additional 0.1 % D-xylose. For biomass production a complex medium was composed, containing: D-xylose or beta xylan 6 g, D-glucose 4 g; amino acid mixture obtained through total hydrolysis of egg white 10 g; $CoCl_2 \cdot 6\,H_2O$ 0.3 g; $MgSO_4 \cdot 7\,H_2O$ 0.5 g; KH_2PO_4 1 g. Sugars were autoclaved separately. Distilled water was added to 1 dm^3 volume and the medium obtained was adjusted to pH 7.0. The yeast biomass was produced in 500 cm^3 Erlenmayer flasks, containing 100 cm^3 medium, incubated at 28 °C up to 120 h on a reciprocal shaker amplitude 20 cm, frequency 2 Hz. At regular time intervals, culture samples were withdrawn and centrifuged at 5000 g. The obtained cell mass was washed twice with phosphate buffer (5 mmol dm^{-3}; pH 8.0) and diluted to a known dry cell weight.

1.4. Enzyme assay

The incubation mixture (1 cm^3) for xylose isomerase assay contained known amount of dry cells (10–50 mg) 122 mmol dm^{-3} D-glucose or another sugar, 0.1 mol dm^{-3} $MgSO_4$ and 1 mmol dm^{-3} $CoCl_2 \cdot 6\,H_2O$. The enzyme reaction was carried out at 70 °C in phosphate buffer (5 mmol dm^{-3}; pH 8.0). At appropriate time intervals the isomerization reaction was interrupted by adding 10 cm^3 0.1 mmol dm^{-3} $HClO_4$. Cells or debris were removed by centrifugation at 10 000 × g and ketose thus formed was quantitatively measured following the modified cysteine-carbazol colorimetric method (Dische & Borenfereund, 1951; Nakamura, 1968). Qualitative detection of D-fructose and its separation from D-glucose were performed by descending paper chromatography on Whatman No. 1 paper, in solvent system aceton–butanol–water (7:2:1 by vol.) and detected with benzidine reagent (Cifonelli & Smith, 1954). D-fructose and D-glucose were determined in a separate experiment radiochemically using (U–^{14}C) D-glucose or D-fructose (Institute for Research, Production and Application of Radioisotopes, Prague) (10 mg; radioactivity 248 MBq). Separation of both hexoses was carried out as described previously. The beta-xylanase activity was assayed in aliquots of the fermentation medium (0.5 cm^3) using a soluble chromolytic substrate (carboxymethyl xylan containing covalently linked Remazol Brillant Blue R, 10 mg; substitution with carboxymethyl groups 0.1 mg; with Remazol Brillant Blue R 0.08 mg). The unreacted chromolytic xylan was precipitated with 1 cm^3 stop solution containing 9 volumes of ethanol and 1 volume of hydrochloric acid (1 mol dm^{-3}). Absorbancy (at 620 nm) of the supernatant corresponding to soluble blue xylooligosaccharides was proportional to xylanase activity (Kuniak & Zemek, 1979).

1.5. Cell immobilization

The biomass obtained in complex medium was concentrated by centrifugation (2500 r.p.m.). The sediment was twice washed in distilled water and freeze-dried. The dried biomass was washed with ethanol and toluene and the cells were permeabilized by action of toluene (70 °C, 1 h) and vacuum dried. The cells were immobilized in polyethyleneimine (Fluka, Switzerland). Polyetheleneimine, molecular weight 45 000, as a 10 % (w/v) aqueous solution (19 g) was mixed with 0.095 g of dried permeabilized cells and to the suspension thus prepared 0.4 cm of 2-chloromethyloxirane was added. The crosslinking reaction was carried out for 10 min at 30 °C and the gel prepared in this way was disintegrated in a mixer, washed with water (100 cm^3), dried and sieved (ZEMEK & KUNIAK, 1983).

The biological purity of the experiment was checked by microscopic and cultivation tests. Growth of the cells was followed nephelometrically and/or by counting the cells in the Bürker chamber.

2. Results and conclusions

Almost 20 % of the tested strains (i.e. 75 strains) of yeast-like microorganisms gave positive reaction on the synthetic selection medium with D-xylose. They were as follows: *Bullera alba, Oospora lactis, Cryptococcus albidus, Dioszegia hungarica, Sporobolomyces salmonicolor, Rhodotorula glutinis, Torulopsis kruisii, Aureobasidium pullulans, Metschnikowia pulcherrima alfa, Candida albicans, C. tropicalis, C. humicola, C. intermedia, C. curvata, C. muscorum, C. parapsilosis, C. solani, C. utilis, C. oregonensis, C. natalensis, C. lusitaniae, C. obtusa, C. stellatoidea, C. mogii, C. shehatae, C. steatolytica, Trichosporon cutaneum, T. fermentans, T. brasicae, T. aculeatum, T. terrestre, T. lutetiae, Lipomyces lipofer, Nadsonia fulvescens, Hormoascus platypodis, Pichia membranaefaciens, Citeromyces matritensis, Kluyveromyces fragilis, Pachysolen tannophilus, Nematospora coryli, Dekkera bruxellensis, Wickerhamia fluorescens, Rhodosporidium teruloides, Leucosporidium scottii, L. capsuligenum, Lodderomyces elongisporus, Wingea robertsii, Filobasidium capsuligenum.*

On the other hand only 9 strains were proved as producers of endo-acting xylanolytic activity (i.e. *Bullera alba, Cryptococcus laurentii, Cryptococcus albidus, Trichosporon cutaneum, Debaryomyces cantarellii, Ambrosiozyma ambrosiae, Leucosporidium scottii, Lodderomyces elongisporus, Wingea robertsii.* All the strains of *Aureobasidium pullulans* tested (i.e. 21) had been found to be β-xylanase producers (ČERNÁKOVÁ et al., 1980).

In respect to the price of D-xylose as an inducer for xylose isomerase we considered in further experiments just those xylose isomerase producers which

were capable of splitting xylan. TAKASAKI and co-workers (1969) have already shown that cheap D-xylose sources such as wheat bran, corncob and chaff, containing xylan, could replace pure D-xylose as an inducer provided that the strains involved produce β-xylanase. Xylan utilizing strains will obviously grow satisfactorily on xylose and xylose-oligosaccharides present in assorted wood liquors and sulphite liquors now being used as a single cell protein substrate and are thus potential xylose-isomerase producers.

Considering the morphology and the semiquantitatively evaluated extent of the isomerisation reaction (paper chromatography) and β-xylanase reaction monitored with the substrate (KUNIAK & ZEMEK, 1979) *Wingea robertsii* was found to be the most suitable xylose isomerase producer. Specific activities of the xylose isomerase production among the xylanase positive strains was 2–4 times higher in *Wingea robertsii* (~1.8 EU per g) than in other strains (0.3–0.8 EU per g).

The strain of *Wingea robertsii* CCY 68-1-1 was inoculated on selective medium and grown for 96 h at 28 °C. For biomass production a complex medium (2 dm^3) was used. In Table 1 cultivation data of the strain on medium with D-xylose and corresponding activities of β-xylanase and xylose isomerase, are demonstrated. In Table 2 growth on complex medium with xylan and in Table 3 on the medium with tamarind (ex *Tamarindus indiaca*), are shown. According

Table 1

Formation of xylose isomerase and beta-xylanase with Wingea robertsii *cells on medium with D-xylose*

Cultivation time (h)	Weight of dry cells (g dm^{-3})	Xylose isomerase activity (EU g^{-1})		Beta-xylanase activity (EU dm^{-3})	
		$\bar{x}$	±s	$\bar{x}$	±s
2	0.01	0		0	
6	0.015	0		0	
12	0.02	0		0	
24	0.038	0		0	
30	0.059	0		0	
36	0.1	0.3	0.004	0.15	0.001
42	0.34	0.6	0.005	0.15	0.001
48	0.6	0.6	0.004	0.15	0.001
60	0.9	0.7	0.005	0.15	0.002
72	1.5	1.4	0.008	0.15	0.001
84	1.5	1.6	0.007	0.15	0.001
96	1.55	1.6	0.007	0.1	0.001
108	1.55	1.6	0.006	0.1	0.001
120	1.55	1.8	0.007	0.05	0.0005
132	1.55	1.8	0.006	0.05	0.0005
144	1.55	1.8	0.007	0.05	0.0005

Enzyme Unit (EU) is understood to mean the xylose isomerase activity to 1.0 μmol fructose formed in 1 s under conditions specified in para. 1.4.

For beta xylanase activity the EU unit corresponds to 1.0 μmol xylose liberated from the chromolytic xylan in 1 s under conditions as specified in para. 1.4.

The values of 10 measurements are described by expressing the attributes in terms of means ($\bar{x}$) and standard deviations ($\pm s$).

Table 2

Formation of xylose isomerase and beta xylanase with Wingea robertsii *cells on medium containing xylan*

Cultivation time (h)	Weight of dry cells (g dm^{-3})	Xylose isomerase activity (EU g^{-1})		Beta-xylanase activity (EU dm^{-3})	
		$\bar{x}$	±s	$\bar{x}$	±s
2	0.01	0		0.01	0.001
6	0.015	0		0.015	0.001
12	0.02	0		0.03	0.002
24	0.036	0		0.05	0.002
30	0.055	0.34	0.004	0.08	0.006
36	0.095	0.4	0.003	0.09	0.006
42	0.10	0.4	0.003	0.09	0.005
48	0.34	0.5	0.003	0.1	0.006
60	0.7	0.7	0.005	0.3	0.009
72	1.6	1.3	0.005	0.4	0.009
84	1.65	1.4	0.006	0.6	0.010
96	1.65	1.5	0.006	0.7	0.015
108	1.65	1.7	0.007	0.9	0.030
120	1.7	1.8	0.007	1.0	0.050
132	1.7	1.8	0.007	1.0	0.040
144	1.7	1.8	0.008	1.0	0.050

Description as in Table 1

Table 3

Formation of xylose isomerase and beta-xylanase with Wingea robertsii *cells on tamarind containing medium*

Cultivation time (h)	Weight of dry cells (g dm^{-3})	Xylose isomerase activity (EU g^{-1})		Beta-xylanase activity (EU dm^{-3})	
		$\bar{x}$	±s	$\bar{x}$	±s
2	0.1	0		0	
6	0.015	0		0	
12	0.02	0		0	
24	0.03	0		0.02	0.001
30	0.04	0.1	0.005	0.5	0.002
36	0.05	0.2	0.010	0.06	0.003
42	0.07	0.3	0.010	0.07	0.003
48	0.1	0.4	0.020	0.08	0.006
60	0.34	0.5	0.020	0.09	0.006
72	0.8	0.7	0.030	0.2	0.009
84	0.96	0.8	0.040	0.25	0.010
96	1.1	0.8	0.040	0.23	0.010
108	1.1	0.9	0.040	0.24	0.011
120	1.1	0.9	0.050	0.25	0.011
132	1.0	0.7	0.050	0.2	0.010
144	1.0	0.6	0.050	0.15	0.011

Description as in Table 1

to the results obtained in absence of xylan a very low xylanolytic activity was observed, the presence of D-xylose in the medium maintained a sufficiently high xylose isomerase activity. Replacement of D-xylose in the medium by xylan resulted in increasing xylanolytic activity, the xylose isomerase activity, however, remained unaltered. When tamarind, a native derivative of cellulose, containing D-xylose was used in the complex medium instead of xylan both xylanase and xylose isomerase production was retarded.

Activity of xylose isomerase reached its maximum after 120 h of cultivation. The *Wingea robertsii* cells were harvested by centrifugation, washed with water and permeabilized as described in Materials and methods.

Permeabilized cells were used for immobilization through encapsulation and covalent bonding in the gel of polyethyleneimine crosslinked with 2-chloromethyloxirane. Activity of xylose isomerase in the immobilized *Wingea robertsii* cells was followed as described in Materials and methods. When starting with (U—^{14}C) D-glucose as a substrate in the incubation mixture (1 cm^3) under conditions as stated containing immobilized *Wingea robertsii* cells (5 mg) the optimal yield of D-fructose was gained after 8 h at 70 °C (pH 8.0). In 8 h it reached 45 % (Table 4).

In a separate experiment instead of (U—^{14}C) D-glucose 10 mg soluble starch (ex potato) labelled with (U—^{14}C) starch (ex *Chlorella sp.*) 248 MBq was used. Before adding the immobilized cells, α-amylase (ex *Bacillus subtilis*, Bolamylase, LIKO-ČSSR; 5 EU) and glucoamylase (3 EU, ex *Endomycopsis bispora*, Prowiko-GDR) were applied. After 20 min preincubation immobilized cells of *Wingea robertsii* (5 mg) were added. After 10 h the D-fructose concentration reached 42 % of the total sugars in the mixture (Table 4).

Two types of glucose isomerase activities have been established. Numerous microorganisms were screened for D-glucose isomerizing activity, but a genuine, specific D-glucose isomerase apparently does not exist. Instead, the enzymes in this category turned out to be either D-xylose isomerase or D-glucose-6-

Table 4

Conversion of D-glucose to D-fructose catalyzed with immobilized Wingea robertsii *cells*

Time	Formation of D-fructose (%)	
	from D-glucose	from the starch hydrolyzate
1	4	2
2	8	4
3	16	6
4	20	12
5	29	18
6	36	27
7	45	35
8	45	42
10	45	42

-phosphate isomerase. The latter activity, however, requires arsenate for the isomerization. As *Wingea* cells are capable of catalyzing aldo-ketose isomerization in absence of arsenate, it can be considered as a proof of the presence of xylose isomerase in the cells. Since the primary market for glucose/fructose syrups is food manufacture the isomerization reaction catalyzed with these yeast-like organisms is more attractive. When using *Wingea robertsii* instead of bacteria producing xylose isomerase (HAMILTON et al., 1974) there is no danger of pathogenicity of the organism and no need to separate toxic substances. These cells seem to be more advantageous from the technological point of view than bacteria or the alkaline non enzymic isomerization of glucose, because of colour, flavour and composition problems (LLOYD et al.,1972; KUČÁR et al., 1981). It is evident that D-fructose can be separated from D-glucose in the reaction mixture as proposed by the German firm C. F. Boehringer for the production of fructose from sucrose (BOEHRINGER & SONS, 1968). In the Boehringer process, sucrose is hydrolyzed to glucose and fructose in a column packed with a sulphonated polystyrene resin which is partially in the calcium form. The glucose and fructose are eluted separately after chromatographic separation on the same column, and fructose may then be crystallized. The concentration step, crystallization of D-glucose and purification of D-fructose from the mother liquors is appreciated, specially when fructose thus obtained should find its use in parenteral solutions for intravenous infusions (WARD, 1976) or as an alcohol de-intoxicant (GORDON, 1971).

Regarding the enzyme pattern of yeasts and yeast-like organisms, according to literary data known it is obviously not of marginal importance that these organisms are able to catalyze the aldo-ketose transformation in such a great extent. Beside the technological aspect ability to isomerize aldoses and ketoses can find its application in taxonomy, too.

Selected permeabilized and immobilized cells of *Wingea robertsii* have already found their use in the Institute for Research, Production and Application of Radioisotopes (Prague, Czechoslovakia) for preparation of (U—^{14}C) D-fructose from the amylolytic hydrolysate of (U—^{14}C) starch (ex *Chlorella vulgaris*, ZEMEK et al., 1983). According to our preliminary results, unspecificity of xylose isomerase of *Wingea robertsii* can result in preparation of such medical formulations as maltoheptulose and lactulose.

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ENDOGENOUSLY OXIDIZABLE POLYPHENOLS OF MANGO, SAPOTA AND BANANA

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The endogenous substrates for oxidation were ellagic acid in mango peel, none in mango pulp, traces of chlorogenic acid and catechin in sapota tissues and dopamine in banana peel and pulp.

The number of oxidizable substrates as well as their extent of browning was very poor in the peel and pulp of all the three tissues. The poor browning is attributed to lack of polyphenol oxidase (PPO) in mango pulp, presence of mostly non-oxidizable phenolics in mango peel and banana and presence of endogenous inhibitors of PPO in sapota tissues. Sapota tissue in the unripe stage contained some PPO inhibitors of non-dialyzable protein type, which disappeared on ripening.

Keywords: endogenous, polyphenolic substrates, fruits

A study of the qualitative and quantitative nature of polyphenols and their involvement in enzymatic browning of avocado and apple tissues (PRABHA & PATWARDHAN, 1980, 1984) has revealed that the concentration as well as the number of substrates involved in the browning of these tissues is high. Fruits like apples, apricots, pears, peaches, plums, cherries and dates are known to undergo considerable browning (LUH & VILLARREAL, 1964; GAJZÁGÓ et al., 1977; MAIER & METZLER, 1965; FUNG & YANKOV, 1971; YANKOV, 1972; VÁMOS & GAJZÁGÓ, 1974). But there are also fruit tissues like banana and mango pulp which undergo comparatively less or no browning. In the case of sapota where the tissue itself is dark brown in colour, browning cannot be visually observed. It is of interest to know the limiting factors that are responsible for poor browning in these fruits. Hence, the nature of oxidizable substrates, the total phenolic content and PPO activity were studied in one variety each of the fruit species.

1. Materials and methods

1.1. The fruits

Mango (*Mangifera indica* badami variety), Sapota (*Achras sapota*, elongated variety) and banana (*Musa sapientum*, robusta variety) were obtained from the local gardens and were allowed to ripen at room temperature.

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1.2. Extraction, quantitative estimation, chromatographic separation and endogenous oxidation of polyphenols

Polyphenols were repeatedly extracted for about 10 min with 90% ethanol followed by 60% simmering ethanol. The extracts were pooled, filtered and concentrated under vacuum at 65 °C. The pigments and lipid matter present in the concentrated extracts were removed by petroleum ether washing, which resulted in clear aqueous layer, and were re-extracted into ethyl acetate and concentrated under vacuum at 35 °C.

Total phenolics and total flavan content in the aqueous extracts were estimated by the standard method (SWAIN & HILLS, 1959). After chromatographic separation the leucoanthocyanidins were located by a specific reagent (ROUX & MAIHS, 1960) and the leucoanthocyanidin spots from the chromatogram run under identical conditions were marked, eluted and estimated as total flavans. The PPO activity was measured by the method described by WONG and co-workers (1971).

Paper-chromatographic separation of polyphenols was done on Whatman No. 3 chromatographic papers by two dimensional chromatography with butanol–acetic acid–water (4 : 1 : 5) and 2% acetic acid solvent systems for directions I and II, respectively. The identification of the polyphenols was done according to the standard procedures (ROUX & MAIHS, 1960).

For the location of oxidizable substrates, one of the two identical paper chromatograms was sprayed with an endogenous PPO preparation extracted from the same tissue. The dark brown spots of the oxidized phenolics were identified by comparison with the chromatogram sprayed with the ferricyanide – ferric chloride reagent.

2. Results

The concentration of total polyphenols, total flavans and leucoanthocyanidins in the peel and pulp tissues of mango, sapota and banana and their PPO activities are presented in Table 1.

2.1. The polyphenols

The phenolic content in the peel was 10 to 12 times higher than that in the pulp. All the phenolics present in both mango peel and pulp were of non-flavan nature. In sapota tissues, 50—70% of the total phenolics were contributed by flavans and more than 90% of the total flavans consisted of leucoanthocyanidins. In banana peel and pulp, 75% of the total phenolics consisted of flavans, while about 95% of the total flavans were contributed by leucoanthocyanidins.

2.2. The polyphenol oxidase (PPO)

The enzyme activity was found to be higher in ripe mango peel than in its unripe stage. In mango pulp, there was no PPO activity at neither the unripe nor the ripe stages. In banana, the activity of PPO in the ripe peel was higher than in the unripe peel and the reverse was found in the banana pulp. In sapota peel and pulp tissues, the PPO enzyme was detected only in the ripe stage.

2.3. Endogenous oxidation

The oxidizable substrates are described in Table 2. Paper chromatograms of polyphenols separated by two-dimensional chromatography and sprayed with endogenous PPO to reveal the oxidizable substrates indicated ellagic acid to be the only browning substrate in mango peel which showed moderate browning inspite of high concentration. In the case of mango pulp, however, no phenolic compound was found to get oxidized when sprayed with endogenous PPO except for traces of gallic acid in unripe mango pulp.

Table 1

Phenolic concentration and PPO activity in peel and pulp of some fruit tissues (g per 100 g)

Fruit tissues	Total phenolics				Total flavans			
	ripe		unripe		ripe		unripe	
	$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s
Mango peel	1.80	0.22	3.10	0.8	Nil	—	Nil	—
pulp	0.08	0.003	0.07	0.004	Nil	—	Nil	—
Banana peel	0.88	0.08	0.95	0.1	0.72	0.06	0.71	0.09
pulp	0.07	0.001	0.06	0.002	0.05	0.01	0.05	0.003
Sapota peel	0.95	0.07	0.99	0.08	0.51	0.08	0.50	0.02
pulp	0.07	0.003	0.06	0.001	0.05	0.002	0.04	0.001

Fruit tissues	Total leucoantho-cyanidins				PPO activity (sp. activity)[a]			
	ripe		unripe		ripe		unripe	
	$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s
Mango peel	Nil	—	Nil	—	590	20	1 050	47
pulp	Nil	—	Nil	—	Nil	—	Nil	—
Banana peel	0.64	0.05	0.63	0.08	813	30	1 400	55
pulp	0.04	0.002	0.04	0.03	18 330	350	11 000	360
Sapota peel	0.48	0.05	0.48	0.04	240	15	840	28
pulp	0.04	0.006	0.04	0.002	Nil	—	680	25

$\bar{x}$ = average of 6 measurements; ±s = standard deviation
[a]: number of measurements = 3

Table 2

Endogenously oxidizable substrates[a] *in peel and pulp tissues of some fruits*

Fruit tissues		Unripe		Ripe	
		Substrates (oxidizable)	Conc. (mg per 100 g)	Substrates (oxidizable)	Conc. (mg per 100 g)
Mango	peel	ellagic acid	45.0	ellagic acid	47.2
	pulp	gallic acid (traces)	0.8	(no trace of oxidation)	0.48
Banana	peel	dopamine	16.0	dopamine	20.0
	pulp	dopamine	1.3	dopamine	0.88
Sapota	peel	nil	—	catechins & chlorogenic acids (traces of oxidation)	—
	pulp	nil	—		—

[a] The phenolics were identified by their R_f values, absorption maximum, specific test and co-chromatography with authentic compounds.
Number of measurements, n = 3

Table 3

Inhibition of PPO aqueous extracts of unripe sapota tissues

	PPO activity units		
	Sapota pulp (ripe)	Apple pulp (unripe)	Avocado pulp (unripe)
Control[a]	0.15	0.34	0.40
+0.05 cm³ of raw sapota extract[b]	0.0	0.0	0.0
+0.05 cm³ of ripe sapota extract	0.14	0.35	0.38
same as 'b' but dialyzed	0.0	0.0	0.0
same as 'b' but treated with TCA and centrifuged	0.14	0.34	0.38

[a] 2 cm³ of 10 mmol catechol + 0.1 cm³ of PPO enzyme from respective fruit tissues
[b] 25 g of sapota fruit were grated and blended by adding 10 cm³ glass distilled water and centrifuged at 10 000 g for 10 minutes. The aqueous supernatant was used as a source of inhibitor

Dopamine was the only oxidizable substrate in the peel and pulp of banana at both unripe and ripe stages. The dopamine concentration was quite low in banana pulp and the brown colouration of the spot was also faint. Leucoanthocyanidins did not undergo any oxidation though they were present in large quantities.

In sapota peel and pulp at the ripe stage, the oxidizable phenolic substrates were chlorogenic acid, catechin and epicatechin. Since these phenolics were found in traces, the brown colouration of the spots was also very faint. Leucoantocyanidins did not undergo browning though they were present in high quantities. In the case of unripe sapota tissues, none of the phenolics were oxidized when sprayed with the endogenous PPO. But the same phenolic substrates in the unripe sapota tissue underwent browning when sprayed with the enzyme extracts of the ripe fruit tissues indicating the disappearance of inhibitors on ripening.

2.4. Endogenous inhibitors in sapota

The aqueous extracts of unripe sapota peel and pulp was found to prevent oxidation of catechol by PPO from several sources indicating the presence of an endogenous PPO inhibitor. However, the aqueous extracts of ripe sapota tissues did not inhibit catechol oxidation (Table 3). Further dialysis of the aqueous extracts of unripe sapota peel and pulp did not help in removing the inhibition of catechol oxidation indicating that the inhibitors are of non-dialyzable nature. However, when treated with TCA and centrifuged the extracts did not exhibit any inhibitory effect on catechol oxidation suggesting that the inhibitor may be protein in nature.

3. Conclusions

In this study, the peel and pulp tissues of mango, banana and sapota were screened for their respective endogenously oxidizable substrates. Previously it had been noticed in the cases of avocado seed, seed coat, pulp and peel that the number of endogenously oxidizable substrates as well as the intensity of the brown colouration on the chromatograms was very high (PRABHA & PATWARDHAN, 1980). But in the present studies the number of oxidizable substrates and also their extent of browning was very low. In the case of mango, though the peel tissue contained a high concentration of a number of phenolics and a high level of PPO enzyme, only one compound identified as ellagic acid showed slight darkening in the presence of enzyme in spite of its high concentration. This indicated that mango peel PPO is not active towards most of the phenolics present in that tissue. Mango pulp lacked PPO enzyme and hence none of its substrates could contribute to enzymatic darkening of that tissue except for traces of gallic acid. The oxidation of this compound by the enzyme from unripe mango pulp could be demonstrated.

In banana peel and pulp, the only endogenous substrate undergoing enzymatic darkening was dopamine. Dopamine was reported to be the oxidizable substrate in banana (GRIFFITHS, 1959).

Low level of oxidation of catechins and chlorogenic acid exhibited by the enzymes of ripe sapota tissue and the inhibition of catechol oxidation by the aqueous extracts of unripe sapota tissue indicated the presence of endogenous inhibitors that disappeared on ripening. Disappearance of peroxidase inhibitors during ripening of mango was reported by MATTOO and MODI (1970). Whether sapota fruit which is by itself brown in colour does undergo browning reactions is not known. The absence of PPO activity in unripe fruits due to the presence of inhibitors and negligibly low concentrations of endogenously oxidizable substrates indicated that sapota, at least in the unripe stage, cannot undergo enzymatic browning.

Leucoanthocyanidins though present in high concentrations did not form endogenous substrates for enzymatic oxidation in any of the tissues studied unlike the leucoanthocyanidins of avocado tissues which participate in endogenous enzymatic darkening (PRABHA & PATWARDHAN, 1980). Though leucoanthocyanidins are generally considered to be poor phenolase substrates, their contribution to non-enzymatic pink discolouration in processed fruits has been demonstrated (RANGANNA & PARPIA, 1974). So the fruit phenolics not oxidizable by endogenous PPO may still undergo a slow non-enzymatic darkening and present problems in fruit processing.

In the prevention of enzymic darkening most control processes depend on inactivation of the enzyme either by heat or by chemical inhibitors. But, in some cases natural control exists as is found in the case of mango pulp which lacks PPO system, or unripe sapota tissue which contains endogenous inhibitors of PPO, or mango peel which contains mostly non oxidizable phenolics. These may be considered as the limiting factors in endogenous browning in these tissues.

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IN VITRO EFFECT OF POLYPHENOLS ON SOME ENZYME SYSTEMS

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The effect of phenolic compounds on a few isolated enzyme systems of fruits was studied under in vitro conditions.

The effect was more pronounced on succinic dehydrogenase (SDH) and peroxidases (POD). Inhibitory effects of chlorogenic acid, catechol and hydroquinone and stimulatory effect of catechin and gallic acid were noticed for SDH. Ferulic, caffeic and p-coumaric acid showed a high activating effect on POD whereas chlorogenic acid, catechin catechol, hydroquinone protocatechuic acid, dopamine and gallic acid inhibited the enzyme. Inhibitory effects of hydroquinone, p-coumaric and ferulic acid was noticed on polyphenol oxidase (PPO) enzyme. There was not much effect of polyphenols on enzymes like amino transferases and ATPases.

Keywords: polyphenols, inhibitors, activators, enzyme systems

Flavonoids have been implicated in several functions of importance like disease resistance due to their antifungal and antibacterial properties (MC CLURE 1975; OLAH & SHERWOOD, 1973). Oxidized phenolics have been shown to be potent inhibitors of pectolytic enzymes which are responsible for easy invasion of fungal pathogens of the soft tissues (SWARDT et al., 1967). IAA oxidase which controls the auxin concentration and in turn growth of plant has been shown to be inhibited by some phenolics and activated by certain other phenolics. (SANO, 1971; RUNKOVA et al., 1972). Polyphenols acting as uncouplers of oxidative phosphorylation (MCCLURE, 1975; STENLID, 1970) and as inhibitors of ion uptake (STENLID, 1962) due to their effect on various enzyme systems have been reported.

In this study data are presented on the effects of several phenolics on some isolated enzyme systems like SDH, POD, PPO and others.

1. Materials and methods

1.1. Mitochondrial isolation

The homogenate from pulp portion of the fruits, viz. apple, avocado, banana and mango was prepared in a medium (pH 7.8) containing 0.4 mol sucrose, 0.2 mol tris, 0.01 mol potassium dihydrogen phosphate, 0.01 mol EDTA, 0.5% (w/v) PVP and 0.05% (w/v) cysteine hydrochloride, as described

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by HULME and co-workers (1964). The homogenate after removing the cell debris and nuclear fraction was subjected to centrifugation at 10 000 × g for 20 min, to sediment mitochondrial fraction. Mitochondrial pellet was suspended in 0.03 mol sucrose after washing once with 0.33 mol sucrose.

1.2. Enzyme assay

SDH activity in the mitochondrial preparations was assayed by the method of HOSKINS and MACKENZIE (1961). The reaction medium containing mitochondria and the inhibitor was equilibrated for 10 min. The reaction was started by sequential addition of dye, substrate and phenazine methosulphate. POD isolated from the acetone powder of mango pulp was measured by the method described by VANCE and SHERWOOD (1976). PPO extracted from the acetone powders of apple, avocado and banana was estimated as described by WONG and co-workers (1971).

2. Results

2.1. Succinic dehydrogenase

The effect of polyphenols on SDH activities of apple, avocado, banana and mango is given in Table 1. Polyphenols had a pronounced effect on SDH from all the fruit tissues studied. At 10^{-3} mol concentration, catechol and hydroquinone showed 100% inhibition of avocado and apple SDH while mango and banana SDH was inhibited to the extent of 70%. Dopamine, chlorogenic acid

Table 1

In vitro effect of phenolics on SDH activity[b] in fruit systems

Polyphenols[a]	Apple		Avocado		Banana		Mango	
	(10^{-3} mol)	(10^{-4} mol)	(10^{-3} mol)	(10^{-4} mol)	(10^{-3} mol)	(10^{-4} mol)	(10^{-3} mol)	(10^{-4} mol)
Nil (control)	100	100	100	100	100	100	100	100
Catechol	0	25	0	18	25	38	28	62
Hydroquinone	0	20	0	15	19	45	0	52
Chlorogenic acid	5	48	10	46	20	50	20	55
Dopamine	29	60	35	63	0	10	38	78
Epicatechin	20	80	15	76	35	82	40	78
Catechin	60	94	65	90	212	119	190	128
Gallic acid	380	147	370	152	385	150	340	152

[a] Resorcinol, *o*, *m* and *p*-cresols, protocatechuic, shikimic, caffeic, quinic, ferulic and *p*-coumaric acids did not have much effect on SDH in apple, avocado, banana and mango

[b] Activities expressed are relative to control value taken as 100

Number of determinations (n) = 3

and epicatechin also inhibited SDH activity in the range of 65–95 % in different fruit tissues. On the other hand, catechin and gallic acid showed high activation of SDH activity of banana and mango. But catechin showed inhibitory effect of about 30–40 % on the SDH of apple and avocado. It was also noticed that the SDH assay medium turned reddish brown upon addition of catechin due to its oxidation by the mitochondrial PPO enzyme from apple and avocado. Hence various inhibitors of PPO (those which did not inhibit SDH) were used in SDH assay medium to prevent catechin oxidation. Among them, sodium diethyl dithiocarbamate was found to be the most suitable and inhibited catechin oxidation to 84 and 100 % at 10^{-4} and 10^{-3} mol concentration, respectively. Hence, the effect of catechin on SDH of apple and avocado was studied with and without the presence of sodium diethyl dithiocarbamate (Table 2). In presence of the compound catechin exhibited twofold activation of SDH activity of apple and avocado at 10^{-3} mol concentration, whereas in the absence of diethyl dithiocarbamate catechin inhibited both avocado and apple SDH activities. Certain compounds did not have any effect on the enzyme.

2.2. Peroxidase

Since mango pulp did not possess PPO activity, the effect of various polyphenols could be studied only in the case of mango pulp (Table 3). At 10^{-3} mol concentration, catechol, protocatechuic acid, tannic acid and hydroquinone exhibited about 70–95 % inhibition of peroxidase activity. Dopamine and gallic acid showed 40 to 60 % inhibition and catechins showed even less inhibitory effect. Certain phenolics did not have much inhibitory effect. *p*-Coumaric, caffeic and ferulic acids showed high activation of POD enzyme, the activation (%) at 10^{-3} mol concentration being 248, 700 and 1500, respectively, for the 3 compounds. Caffeic and ferulic acids even at as low a concentration as 10^{-6} mol exhibited activation of peroxidases.

Table 2

Catechin effect on SDH in apple and avocado mitochondria with and without the presence of diethyl dithiocarbamate[a] (DEDTC)

	Apple	Avocado
Catechin at 10^{-3} mol conc. without sodium DEDTC	−40	−35
Catechin at 10^{-3} conc. with sodium DEDTC	+120	+135
Catechin at 10^{-4} mol conc. without sodium DEDTC	−6 to + 10	−6
Catechin at 10^{-4} mol conc. with sodium DEDTC	+30	+40

[a] Sodium DEDTC was taken at the concentration of 10^{-4} mol × 5
Values are expressed as percent inhibition and activation
− = inhibition + = activation
Number of determinations (n) = 3

Table 3

In vitro effect of polyphenols on peroxidase of mango pulp

Phenolics	Activity retained over the control				
	10^{-3} mol	5×10^{-4} mol	10^{-4} mol	10^{-5} mol	10^{-6} mol
Control	100	100	100	100	100
Catechol	28	38	—	—	—
Hydroquinone	4	12	—	—	—
Chlorogenic acid	80	82	—	—	—
Catechin	70	75	—	—	—
Epicatechin	75	80	—	—	—
Dopamine	48	40	—	—	—
Gallic acid	56	64	—	—	—
p-Coumaric acid	248	125	—	—	—
Caffeic acid	700	482	296	132	104
Ferulic acid	1500	1404	1044	196	120
Protocatechuic acid	32	56	—	—	—
Tannic acid	24	25	—	—	—
Salycilic, shikimic and quinic acids	100	100	100	100	100

— = not determined
Number of determinations (n) = 3

Table 4

In vitro effect of phenolics on PPO activity of some fruits

Phenolics	Activity retained over the control					
	Apple		Avocado		Banana	
	10^{-3} mol	10^{-4} mol	10^{-3} mol	10^{-4} mol	10^{-3} mol	10^{-4} mol
Control	100	100	100	100	100	100
Hydroquinone	18	35	15	48	20	54
Pyrogallol	20	35	25	40	22	50
α-Naphtol	15	46	15	50	18	42
p-Coumaric acid	70	—	75	—	60	—
Ferulic acid	65	—	68	—	75	—
Other phenolics[a]	100	—	100	—	100	—

[a] benzoic, salicylic, cinnamic acid, resorcinol, *p*-chlorophenol, *m*, *o* and *p*-cresols
— = not determined
Number of determinations (n) = 3

2.3. Polyphenol oxidase

The effect of some non oxidizable phenolics on some PPO systems was inhibitory (Table 4). Hydroquinone, pyrogallol and α-naphthol showed about 80 % inhibition whereas p-coumaric and ferulic acids exhibited inhibition around 30 %. Certain phenolics did not have any inhibitory effect.

2.4. Other enzymes

The effect of polyphenols on amino transferases and ATP'ases was not much and hence is not presented in a table.

3. Conclusions

Several phenolics have been reported to inhibit enzymes like malate dehydrogenase, glutamate decarboxylase, glycerol dehydrogenase, hexokinase and ribonuclease (McCLURE, 1975). The results of the present study reveal that catechol and hydroquinone are potent inhibitors of SDH whereas catechin and gallic acid are good activators of the enzyme. Dopamine, chlorogenic acid and epicatechin exhibited considerable inhibition. Catechin and epicatechin being isomers of the same compound showed opposite effect on SDH activities, catechin being an activator and epicatechin an inhibitor of the enzyme. Further, it is inferred from the results that non oxidized catechin has an activating effect on SDH activities and oxidized catechin has an inhibitory action. It must be noted here that some of the compounds which affected SDH activities are endogenously present in fruits. For instance catechin and epicatechin are present in both apple and avocado (VAN BUREN, 1970; PRABHA & PATWARDHAN, 1980). Dopamine, chlorogenic acid and gallic acid are the endogenous polyphenols in banana, apple and mango flesh, respectively (VAN BUREN, 1970). Polyphenols were shown to affect mitochondrial metabolism like respiration, ion uptake and oxidative phosphorylation (McCLURE, 1975). Our experiments revealed that polyphenols have a pronounced effect on SDH which is a key mitochondrial enzyme.

As for POD many events of importance like fruit ripening and metabolism of plant hormones are linked with the enzyme. The activating effect of some monophenols and the inhibitory effect of some dihydroxyphenols on POD observed in our experiment coincide with similar observation on IAA oxidase activity which is a peroxidase controlling the level of IAA, a plant growth regulator (SANO, 1971; RUNKOVA et al., 1972).

The inhibitory effect of some non-oxidizable polyphenols on PPO enzyme systems may be of significance in controlling browning.

Different polyphenols showed varied effect on enzyme systems. For instance, the activity of the enzymes like ATP'ases and aminotransferases was not much affected by the polyphenols whereas a pronounced effect of some of these compounds on SDH and POD was observed. It is also interesting to note that catechin and gallic acid which showed activating effect on SDH are inhibitory to POD. Further, p-coumaric, caffeic and ferulic acids which showed a high activating effect on POD have not had any noticeable effect on SDH activities and are inhibitory to PPO enzymes.

Thus, our studies have indicated that while a major part of phenolic compounds inhibited to various degrees the enzyme systems studied, some polyphenols exerted activating effect on the same enzyme systems.

All the enzymes under investigation i.e. SDH, POD and PPO are metallo enzymes containing either iron or copper. Various o-dihydroxy and vicinal trihydroxy phenols are known to chelate metals as reported by VAN BUREN (1970). It is likely that the inhibition noticed in the present study is due to metalchelation. WALKER and WILSON (1975) have shown that compounds like *p*-coumaric and ferulic acids act as non competitive on particulate and as competitive inhibitors on soluble enzyme systems from apple. VÁMOS-VIGYÁZÓ (1981) has also reviewed the effect of polyphenolic inhibitors and activators of PPO enzyme systems.

On the other hand some polyphenols have been shown to act as activators also. HYODO and URITANI (1966) observed increase in o-diphenolase activity during incubation of sliced sweet potato. According to these authors, protein association and dissociation is likely to be involved in this process.

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ION EXCHANGE COLUMN CHROMATOGRAPHY FOR THE DETERMINATION OF KERATIN IN MEAT MEALS

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Attempts were made to determine the keratin content in meat meals by different methods of ion-exchange chromatography. The chemical composition of the analysed feather and meat as well as feather meal and meat meal samples did not permit the determination of keratin. On the other hand the amino acid composition, particularly the cysteine content and the indices Cys per Lys × His × Met and Cys × Ser per Lys × His × Met calculated from the amino acid composition permitted the detection of 2–3% feather meal mixed to meat meal. Even 1–2% previously non-hydrolyzed feather meal could be detected by protein fraction analysis.

Keywords: ion-exchange column chromatography, keratin determination, keratin

Just like other structural proteins keratin, the protein of feather is also insoluble and highly resistant to all substances which do not chemically attack and destroy it. The most expedient method of solubilization is the oxidation or reduction of the disulfide bridges (ELŐDI, 1972). The structure of solubilized keratin lends itself to analysis. In the course of keratin analysis it was established that it breaks down into two fractions one of which is rich in sulfur while the other is poor. The fraction containing little sulfur consists of fibrillar molecules forming fibers while the fraction of high sulfur content is globular. In keratin the fibers consist of fibrillar molecules bound together by the globular elements of high sulfur content. The diet of the animal affects the keratin component of high sulfur content. With increasing sulfur content of the fodder increases the quantity of the binding protein and at the same time changes the quality of the keratin containing elements (feather, claw, fur, etc.). The substantial difference between the structural proteins of feather and fur is caused by difference in their development. Fur grows under pressure, therefore it is thread-like (containing mostly alfa-keratin) while feather grows freely and becomes therefore ramified (consisting mainly of beta-keratin) (KÁLLAI, 1972).

Fur and feather protect the animal against the rigours of weather. Proteins in materials evolved in the course of phylogenesis to perform some special task differ not only in their function but in their amino acid composition as well from that of proteins used in animal feeding. BAGDY (1983) found that

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the proteins of feather and fur are not suitable for animal feeding even when decomposed. HUNGARIAN STANDARD (1980) specifies that product of 58 or 62% raw protein content must not contain feather meal (keratin protein). Mixed animal protein meals should not contain more than 10% keratin at most. The methods used at present in Hungary are not suitable to give exact information on the feather meal content in meat meals. This demand observed by the authors and supported by BOKORI (1984) as well, incited us to attempt to detect the feather meal content in meat meals. We summarized our results in the following.

1. Materials and methods

1.1. Materials analysed

Prior to starting on the analysis of meat and feather meals as by-products of the meat industry we looked for model materials which would permit of discovering the differences characterizing meat and feather meals. While studying these materials substances may be found which are present in minute quantities or not at all in one of the materials and in abundance in the other. Thus, prior to investigating meat meal and feather meal originating from the meat industry the composition of meat and feather meal free of every industrial intervention was established.

The model materials investigated were the following:

— Round of beef, originating from 5 different animals dried in a drying oven of 60 °C for 24 h. After drying the meat was defatted by Soxhlet extraction for 6 h with petroleum ether of 60–80 °C boiling point;

— The mixture of white and pigmented (brown) hen's and white dove's feather, 5 each, obtained from the Experimental Plant of our institute. The feathers were dried at 60 °C for 24 h, then cut up with scissors into bits of 2–3 mm and ground in a microculatti attrition mill (Janke & Kunkel KG IKA Werk, Staufen, FRG).

When model investigations were finished the following meat and feather meals obtained from ATEV (Animal Protein Feedstuff Producing Company, Budapest) were analysed:

— meat meal with some feather, marked H_1 (Debrecen),
— meat meal, marked H_2 (Tököl),
— traditional feather meal manufactured on a desinfector, marked T_1,
— improved, hydrolyzed feather meal, marked T_2.

1.2. Methods

The dry matter, crude fat, crude ash contents, the degree of acidity, peroxide number and the digestibility of the experimental material were determined according to the related feed standards. The crude protein content was

determined on the Kjel-Foss rapid nitrogen analyser (Foss Electric, Denmark). The amino acid composition and protein fraction analysis was carried out with the LKB 4101 type automated amino acid analyser (LKB Biochrom Ltd., England). The protein was hydrolysed by MOORE and STEIN's method (1963). To determine the cysteine in the form of cysteic acid of high sulfur content the rapid method developed by the authors (CSAPÓ & WÖLLER, 1980) for materials of high protein content and modified for different feedstuffs (CSAPÓ, 1982), was used.

Evaluation of the amount of individual amino acids in the samples was carried out by the comparison of the regions below the peaks in chromatograms of the samples and the peak regions of standard amino acid solutions. The chromatograms were evaluated also by the method of the authors (CSAPÓ et al., 1984) developed for the elimination of losses originating from the breakdown and oxidation of ninhydrin referring to the inner cysteic acid standard.

The feather meal hydrolysis for protein fraction analysis was carried out as follows: 10–40 mg feather meal were weighed in 10 cm^3 medical ampoules each and oxidized with performic acid prepared according to HIRSCH (1956) at 50 °C for 30 min. The ampoules were immediately cooled to –55 °C and lyophilized at –55 °C till dry. The residue was dissolved in 1 mol NaOH and stored frozen at –25 °C till used in analysis (cca 1 week). When using it in protein fraction analysis, it was heated to 30 °C and its pH was set at 2.2 with 6 mol HCl. The solution of pH 2.2 was applied to the ion exchange column of the amino acid analyser, and analysis was carried out as usual.

1.3. Statistical analysis

To establish differences or identities between the components and calculated indices of the various mixtures, meat meals and feather meals significance tests were carried out. Means and standard deviations as well as comparison means (*t* test) were calculated on a HT PTK-1050 type pocket computer (Híradástechnika Szövetkezet, Hungary). One variable variancia analyses and the homogeneity of variances (Bartlett test) were calculated on a HT PTK 1096 type pocket calculator. Only those data were compared which had been previously analysed for homogeneity.

2. Results

Table 1 contains the composition data of the different meat and feather, meat meal and feather meal samples.

Table 2 contains the amino acid compositions calculated in g amino acid per 100 g sample (white feather, pigmented feather of hens, dove feather) of the different samples, while Table 3 the amino acids per 100 g protein.

Table 1

Chemical composition of the different meat and feather, meat meal and feather meal samples analysed

Sample	Dry matter	Crude protein	Crude fat	Crude fibre	Crude ash	Extracts free from N	Fat quality[a]	
	(g per 100 g)						peroxide number	degree of acidity
Beef (dried)	94.3	79.5	10.8	—	4.00	—	1	1
White hen's feather	93.4	90.8	1.1	—	1.49	—		
Pigmented hen's feather	93.1	91.2	0.8	—	1.05	—		
White dove feather	92.3	90.9	0.9	—	0.47	—		
H_1	94.4	66.1	6.9	0.4	20.0	1.0	1	78
H_2	91.9	61.0	17.2	0.3	13.4	—	1	105
T_1	94.1	84.7	6.4	—	3.0	—	1	108
T_2	94.3	82.7	8.6	—	3.0	—	1	63

H_1 = meat meal with feather (Debrecen); H_2 = meat meal (Tököl)
T_1 = feather meal manufactured on traditional desinfector; T_2 = improved hydrolyzed feather meal
[a] = was not measured in feather

Table 2

Amino acid composition of beef and feather
(g amino acid per 100 g sample)

Amino acid	Beef		White hen's feather		Pigmented hen's feather		White dove feather		Average in feather
	$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s	
Asp	7.67	0.22	5.81	0.24	5.91	0.20	6.83	0.21	6.18
Thr	3.44	0.10	3.91	0.11	4.19	0.13	3.83	0.12	3.98
Ser	3.15	0.11	9.31	0.26	9.38	0.27	8.78	0.32	9.16
Glu	12.44	0.35	8.38	0.26	8.62	0.25	8.53	0.24	8.51
Pro	5.18	0.19	10.73	0.31	10.39	0.35	11.11	0.37	10.74
Gly	3.94	0.12	5.64	0.19	5.50	0.16	6.07	0.18	5.74
Ala	4.34	0.14	4.06	0.12	3.83	0.10	4.52	0.13	4.14
Cys	0.54	0.04	6.33	0.18	6.92	0.23	5.90	0.19	6.38
Val	4.00	0.14	5.61	0.16	5.86	0.17	5.37	0.15	5.61
Met	2.79	0.11	0.40	0.04	0.42	0.02	0.39	0.03	0.40
Ile	4.02	0.18	3.59	0.14	3.85	0.10	3.71	0.11	3.72
Leu	6.38	0.24	6.84	0.27	6.76	0.21	6.81	0.22	6.80
Tyr	2.84	0.14	2.17	0.09	2.19	0.10	3.81	0.12	2.72
Phe	3.14	0.13	4.26	0.17	4.11	0.14	3.90	0.19	4.09
Lys	6.59	0.24	1.12	0.04	0.85	0.03	0.71	0.05	0.89
His	2.44	0.08	0.49	0.03	0.57	0.02	0.64	0.05	0.57
Arg	5.12	0.22	5.30	0.15	5.69	0.21	5.12	0.23	5.37
NH_3	1.28	0.09	1.12	0.07	1.50	0.05	1.55	0.06	1.39
Total	79.30	2.39	85.07	2.42	86.54	2.50	87.58	2.14	86.40
N×6.25	79.5	0.79	90.8	0.71	91.2	0.81	90.9	0.64	91.0

$\bar{x}$ = average value; ±s = standard deviation

Table 3

Amino acid composition in beef and feather
(g amino acid per 100 g protein)

Amino acid	Beef		White hen's feather		Pigmented hen's feather		White dove feather		Average in feather
	$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s	
Asp	9.6	0.30	6.8	0.26	6.9	0.25	7.8	0.29	7.2
Thr	4.3	0.14	4.7	0.15	4.9	0.17	4.4	0.19	4.7
Ser	4.0	0.15	10.0	0.35	10.8	0.34	10.0	0.37	10.3
Glu	15.7	0.51	9.9	0.33	10.0	0.27	9.8	0.30	9.9
Pro	6.6	0.21	12.6	0.43	12.1	0.40	12.6	0.39	12.4
Gly	4.9	0.17	6.7	0.21	6.4	0.24	6.9	0.20	6.7
Ala	5.5	0.16	4.8	0.17	4.5	0.14	5.1	0.19	4.8
Cys	0.7	0.02	7.5	0.33	8.0	0.35	6.8	0.21	7.4
Val	5.0	0.16	6.7	0.21	6.9	0.25	6.1	0.20	6.6
Met	3.5	0.13	0.5	0.02	0.5	0.03	0.5	0.02	0.5
Ile	5.0	0.16	4.2	0.17	4.4	0.14	4.2	0.15	4.3
Leu	8.0	0.21	8.1	0.23	7.8	0.20	7.7	0.24	7.9
Tyr	3.6	0.11	2.6	0.10	2.6	0.17	4.3	0.18	3.2
Phe	4.0	0.17	5.0	0.16	4.8	0.19	4.5	0.21	4.8
Lys	8.4	0.31	1.3	0.05	1.0	0.03	0.8	0.02	1.0
His	3.1	0.12	0.6	0.02	0.7	0.03	0.9	0.03	0.7
Arg	6.5	0.20	6.0	0.22	6.6	0.28	5.9	0.19	6.2
NH_3	1.6	0.08	1.8	0.06	1.7	0.09	1.8	0.07	1.8

The cysteine, methionine, lysine and histidine contents of the samples of different meat meal and feather meal mixtures, calculated in g amino acid per 100 g sample are summarized in Table 4. The Cys per Lys × His and Cys per Lys × His × Met indices calculated from the amino acid compositions of the various mixtures are given also in Table 4.

In Tables 5, 6 and 7 the results of significance tests as calculated from data in Table 4 are summarized.

After establishing the amino acid composition of meat and feather samples, the composition of meat meals and feather meals were determined and summarized in Table 8.

The results of the ion exchange column chromatography of meat and feather samples and their mixtures of different proportions, oxidized in performic acid and subsequent dissolution in 1 mol NaOH solution are contained in Fig. 1 and Fig. 2.

Figure 1/a shows the protein fraction analysis of the meat sample, Fig. 1/b the meat containing 25% feather, Fig. 1/c the 50 : 50% mixture of meat meal and feather meal, Fig. 1/d the 25 : 75% mixture of meat meal and feather meal and Fig. 1/e that of the feather sample after oxidation with performic acid and dissolution in 1 mol NaOH solution.

Table 4

Cysteine, methionine, lysine and histidine in different

Amino acid	Beef		2% feather 98% meat		5% feather 95% meat	
	$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s
Cys	0.54	0.04	0.66	0.03	0.83	0.03
Met	2.79	0.11	2.74	0.08	2.67	0.08
Lys	6.59	0.24	6.48	0.22	6.31	0.19
His	2.44	0.08	2.40	0.09	2.37	0.07
Indices						
$\frac{\text{Cys}}{\text{Lys} \times \text{His}} \times 100$	3.358	0.22	4.244	0.23	5.597	0.25
	2.905	—	3.776	—	5.086	—
Limit value	3.870		4.772		6.163	
$\frac{\text{Cys}}{\text{Lys} \times \text{His} \times \text{Met}} \times 100$	1.204	0.10	1.549	0.10	2.096	0.13
	1.002	—	1.339	—	1.849	—
Limit value	1.444		1.467		2.378	

Table 5

Significance tests between the cysteine and methionine contents of meat and feather as well as their mixtures of different proportion

		Cysteine						
		Beef	2	5	10	20	50	Feather
		feather %						
Methionine feather %	Beef	—	5.37 ***	12.97 ***	23.32 ***	40.86 ***	60.62 ***	64.03 ***
	2	0.82 Ø	—	8.96 ***	21.02 ***	40.27 ***	59.97 ***	63.24 ***
	5	1.97 Ø	1.38 Ø	—	13.42 ***	33.75 ***	56.34 ***	61.36 ***
	10	3.78 **	3.56 **	2.17 Ø	—	20.25 ***	48.37 ***	57.56 ***
	20	8.23 ***	9.05 ***	7.57 ***	5.26 ***	—	35.00 ***	50.65 ***
	50	22.02 ***	27.02 ***	25.36 ***	22.75 ***	18.46 ***	—	29.20 ***
	Feather	46.87 ***	61.24 ***	59.41 ***	56.53 ***	56.08 ***	46.02 ***	—

(Significance levels: Ø = $P \geq 5\%$; ** = $P \leq 1\%$; *** = $P \leq 0.1\%$)

mixtures of beef and feather (g amino acid per 100 g sample)

10% feather 90% meat		20% feather 80% meat		50% feather 50% meat		Feather	
$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s	$\bar{x}$	±s
1.13	0.04	1.71	0.05	3.46	0.10	6.38	0.20
2.56	0.08	2.31	0.07	1.60	0.05	0.40	0.03
6.02	0.17	5.45	0.13	3.75	0.12	0.89	0.04
2.26	0.07	2.05	0.06	1.51	0.05	0.57	0.03
8.306	0.37	15.305	0.61	38.190	1.25	1257.6	32.41
7.558	—	14.099	—	33.730	—	1107.5	—
9.148		16.624		43.336		1433.6	
3.244	0.18	6.626	0.30	61.104	1.99	3144.1	83.8
2.863	—	5.924	—	55.655	—	2575.6	—
3.689		7.422		67.172		3874.5	

Amino acid	Results of one variable analysis: Degree of freedom in numerator	Degree of freedom in denominator	Calculated F value	Critical value of F at P = 0.1% level
Cys	6	28	2535	5.24
Met	6	28	675.6	5.24
Lys	6	28	753.8	5.24
His	6	28	526.1	5.24
Cys × 100 per Lys × His	6	28	7552	5.24
Cyx × 100 per Lys × His × Met	6	28	6784	5.24

Table 6

Significance tests between the lysine and histidine contents of meat and feather as well as their mixtures of different proportion

Histidine, feather %	Lysine: Beef	2	5	10	20	50	Feather
	feather %						
Beef	—	0.76 Ø	2.04 Ø	4.33 **	9.34 ***	23.67 ***	52.38 ***
2	0.74 Ø	—	1.31 Ø	3.70 **	9.01 ***	24.36 ***	55.90 ***
5	1.47 Ø	0.59 Ø	—	2.54 *	8.35 ***	25.47 ***	62.42 ***
10	3.79 **	2.75 *	2.48 *	—	5.96 ***	24.39 ***	65.68 ***
20	8.72 ***	7.24 ***	7.76 ***	5.09 ***	—	21.48 ***	74.96 ***
50	22.04 ***	19.33 ***	22.35 ***	19.50 ***	15.46 ***	—	50.56 ***
Feather	48.95 ***	43.13 ***	52.85 ***	49.62 ***	49.33 ***	36.05 ***	—

(Significance levels: Ø = P ≥ 5%; * = P ≤ 5%; ** = P ≤ 1%; *** = P ≤ 0.1%)

Table 7

Significance tests between the Cys×100 per Lys×His and Cys×100 per Lys×His×Met factors of meat and feather and their mixtures of different proportion

Cys×100 per Lys×His×Met, feather %	Cys×100 per Lys×His: Beef	2	5	10	20	50	Feather
	feather %						
Beef	—	6.22 ***	15.03 ***	25.70 ***	41.19 ***	61.37 ***	86.53 ***
2	5.45 ***	—	8.91 ***	20.85 ***	37.94 ***	59.72 ***	86.47 ***
5	12.16 ***	7.46 ***	—	13.57 ***	32.93 ***	57.17 ***	86.38 ***
10	22.15 ***	18.41 ***	11.56 ***	—	21.94 ***	51.26 ***	86.18 ***
20	38.34 ***	35.90 ***	30.98 ***	21.62 ***	—	36.79 ***	85.69 ***
50	67.22 ***	66.83 ***	66.16 ***	64.75 ***	60.53 ***	—	84.07 ***
Feather	83.86 ***	83.35 ***	83.84 ***	83.81 ***	83.72 ***	82.24 ***	—

(Significance level: *** = $P \leq 0.1\%$)

Table 8

Amino acid composition of meat meal and feather meal

Amino acid	Meat meal with some feather meal (Debrecen)		Meat meal (Tököl)		Feather meal manufactured on traditional desinfector		Improved hydrolyzed feather meal	
	A	B	A	B	A	B	A	B
Asp	5.29	8.0	4.58	7.6	5.17	6.0	5.35	6.4
Thr	2.56	3.9	2.03	3.4	3.65	4.2	3.50	4.2
Ser	3.30	5.0	3.37	5.6	8.48	9.8	8.30	10.0
Glu	8.08	12.3	7.49	12.4	8.77	10.0	8.89	10.7
Pro	6.14	9.3	5.68	9.4	8.01	9.3	8.01	9.7
Gly	6.85	10.4	5.49	9.1	6.14	7.1	6.48	7.8
Ala	5.74	8.7	4.60	7.6	5.06	5.9	3.74	4.5
Cys	0.86	1.3	0.83	1.4	6.06	7.0	3.84	4.6
Val	3.88	5.9	4.06	6.7	7.34	8.5	6.94	8.4
Met	1.47	2.2	1.19	2.0	0.63	0.7	0.81	1.0
Ile	2.52	3.8	2.48	4.1	3.49	4.0	4.34	5.2
Leu	4.32	6.6	5.34	8.8	7.80	9.0	6.85	8.3
Tyr	1.78	2.7	2.05	3.4	2.47	2.9	2.68	3.2
Phe	2.44	3.7	2.69	4.4	4.13	4.8	3.99	4.8
Lys	3.62	5.5	2.54	4.2	1.50	1.7	1.78	2.1
His	1.44	2.2	1.05	1.7	0.57	0.7	0.63	0.8
Arg	4.85	7.4	4.03	6.7	5.66	6.5	5.71	6.9
NH_3	0.81	1.2	1.04	1.7	1.49	2.0	1.13	1.4
Total	65.95	100.1	60.54	100.2	86.42	100.1	82.97	100.0
N×6.25	66.1		61.0		84.7		82.7	

(A = g amino acid per 100 g sample, B = g amino acid per 100 g protein)

To prove that our method is suitable to detect minute amounts of feather meal in the meat meal samples we carried out the protein fraction analysis of meat meal samples containing 1.25% (Fig. 2/a), 2.5% (Fig. 2/b), 5% (Fig. 2/c), 10% (Fig. 2/d) and 20% (Fig. 2/e) feather meal, respectively. These results are shown in Fig. 2.

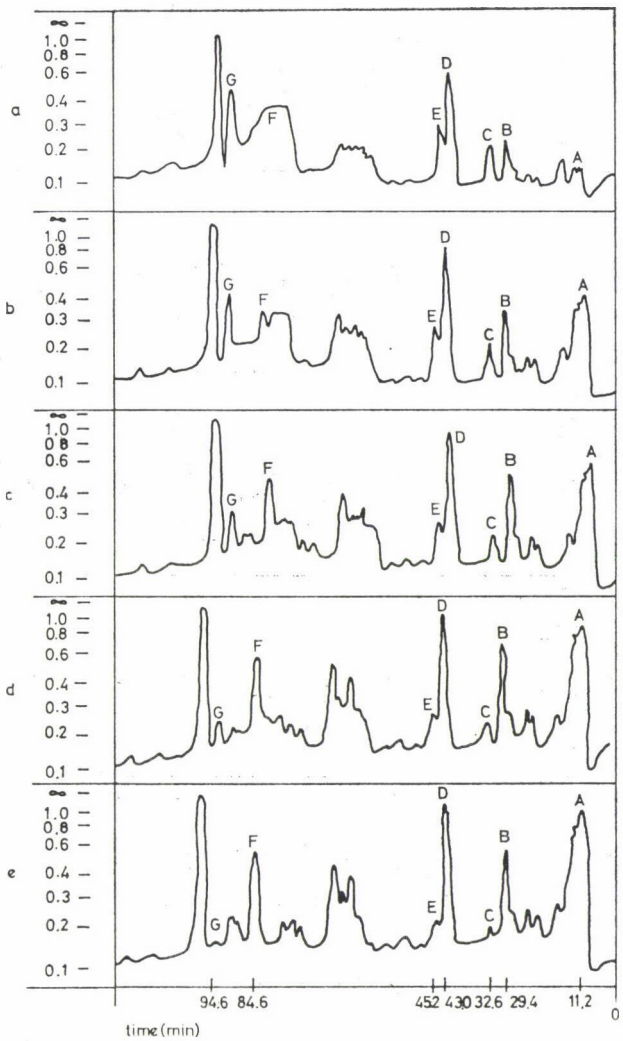

Fig. 1. Protein fraction analysis I. a: Meat; b: Meat with 25% feather; c: 50% meat 50% feather; d: 25% meat 75% feather; e: Feather

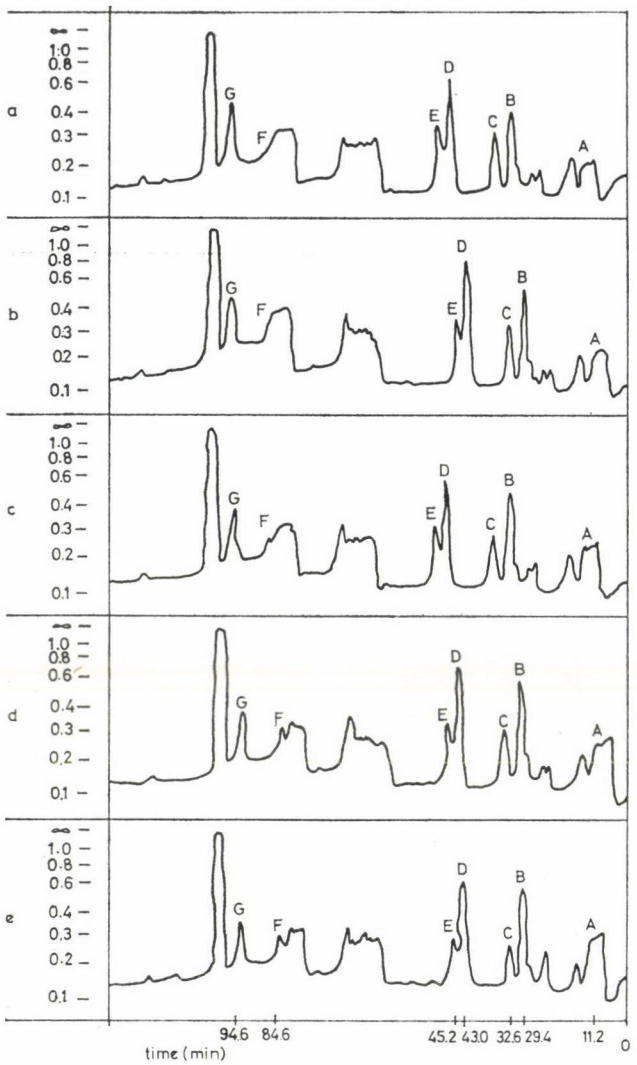

Fig. 2. Protein fraction analysis II. a: 1.25% feather added to meat; b: 2.5% feather added to meat; c: 5.0% feather added to meat; d: 10.0% feather added to meat; e: 20.0% feather added to meat

3. Conclusions

As it can be seen in Table 1 the crude protein content of feather is higher by about 11%, the crude fat content by 10% than that of meat, while the crude ash content is lower by about 3%. It appears to be a significant difference that white hen's feather contains by about 0.5% higher crude ash than the pigmented feather and about 1% higher than dove's feather. In carrying out

the measurement care was taken to take the sample from as many individuals as possible and to take equal proportions of the different types of feather (down, covering and quill feather). The protein content of the feather-meals investigated was about 15–20% higher than that of meat meals and about 6–10% lower than that of feathers. The ash content of meat meals is about 5–7-fold of that in feather meals, while that of the feather meals is about 3-fold of the ash content of feather. Neither of these components gave a reliable information on the feather meal content of the meat meals.

The average cysteine content (Table 2) of feather was found to be 11–12 times higher, while the serine content about 3 times higher than that of meat. On the other hand, the methionine content of meats was seven-fold, the lysine content 7.5-fold, the histidine content more than 4-fold of that of feather. The same components as measured in meat and feather meals showed the following values: feather meals contained about 5–7 times more cysteine, 2.5 times more serine than meat meals. Meat meals, on the other hand, contained about twice as much methionine, lysine and histidine than feather meals. Data obtained for feather and meat meals show a good agreement with the data reported by HEGEDÜS and co-workers (1984) the only exception being lysine, of which the meat meals here investigated had much lower contents. In spite of the fact that the differences in the compositions of amino acids were in every case significant at $P \leq 0.1\%$ level, the minute amounts of feather meal in the meat meal can be detected by measuring the cysteine content or using an index formed from the amino acids above enumerated. The results contained in Table 4 as well as the results of significance tests utilizing these data (Tables 5, 6 and 7) show unambiguously that the addition of 2% feather meal to the meat meal increases the cysteine content of the latter at $P \leq 0.1\%$ significance level. The values for methionine, lysine and histidine show a difference at $P \leq 1\%$ significance level if 10% feather meal is mixed to the meat meal. The indices Cys per Lys × His, Cys per Lys × His × Met or Cys × Ser per Lys × × His × Met show – as it can be well observed from the limit values of the indices – that the addition of 2% feather meal to meat meal causes a significant difference ($P \leq 0.1\%$) in the indices.

If not the model meat and feather are used but the meat meal and feather meal manufactured by the industry then the addition of 4% feather meal can be detected on the basis of the cysteine content (significant at the $P \leq 1\%$ level). The amino acid compositions as measured in feather and feather meal agree on the whole with those reported by WÖHLBIER and TRAN THU (1977) except for cysteine and serine. The values found by them are 1.3–1.4-fold higher than ours. The protein fractions of meat, feather dissolved by oxidation with performic acid and subsequent addition of 1 mol NaOH solution, and of their mixtures of different proportions as obtained by ion exchange column chromatography are shown in Figs. 1 and 2. The R_f value of peak A is 0.092

and its maximum appears 11.2 min after the starting of the analysis. The same R_f values for peak *B:* 0.240 and 29.4 min, for peak *C:* 0.266 and 32.6 min, for peak *D:* 0.351 and 43.0 min, for peak *E:* 0.369 and 45.2 min, for peak *F:* 0.691 and 84.6 min and for peak *G:* 0.773 and 94.6 min, respectively. On comparing the protein fractions of meat and feather it is striking that the region under peak *A* for the feather (height of peak by width as measured at half height) is about 15-fold of that measured for meat. The regions under peaks *B* and *C* are about 10 times larger in feather than in meats, while 7 times larger under peaks *D* and *E*. Fraction of peak *F* is practically missing from meat, while peak *G* is missing from feather. In a mixture containing 25% feather peak *A* substantially increases in comparison to that in 100% meat, peaks *B* and *C* are larger, too. The proportion of peaks *D* and *E* increases as well, peak *F* appears on the chromatogram, while peak *G* decreases. In mixtures of 50–50% and 75 to 25% feather and meat the shift of the peak regions in the direction of that of feather is even more striking. As it can be seen in Fig. 2 peak *A* increases already upon admixing 1.25% feather to meat (the value in the chromatogram increases from 17.40 for meat to 20.09 g for the mixture). The proportion of peak *B* and *C* increases (from 0.96 for meat to 1.47 for the mixture). The proportion of peaks *D* and *E* does not change substantially, peak *F* is too small to be evaluated and the decrease in peak region *G* is minimal. When 2.5% feather is admixed peak region *A* increases to 23.67, while the proportion of peaks *B* and *C* to 1.60. The reduction of peak *G* is still minimal and peak *F* cannot be evaluated. On admixing 10% feather peak *A* increases to 31.84, the proportion of peaks *B* to *C* to 2.03. Peak *G* is reduced significantly, while peak *F* becomes evaluable.

By increasing still further the proportion of feather in the mixture the shifts are more and more striking. 25% feather increases peak *A* to 60.31, the proportion of peak *B* to *C* to 2.63. Peak *F* can be evaluated and the region under peak *G* is reduced significantly.

Results discussed above show that the region under peak *A* or the proportion of peak *B* to *C* suffice to determine whether the meat contains more feather than 2.5% or not. To make the determinations more exact the differences between peak regions as expressed in indices can be used. The value of index $A \times B \times F$ for meat is 78, for feather 91 614. The value of index $A \times D \times F$ for meat is 343, for feather 275 408, that of index $A \times F$ per G for meat 0.74, for feather 3778, that of index $A \times D \times F$ per $E \times G$ for meat 1.77, for feather 62 593 and finally that of index $A \times B \times D \times F$ per $C \times E \times G$ for meat 1.69, for feather 595 001.

If we use instead of pure meat and feather meat meal and feather meal the following changes can be observed: with meat meal from Tököl the value of peak *A* and the proportion of peaks *B* and *C* is somewhat higher than with pure meat, there is no difference in peaks *D*, *E* and *G*, peak *F* cannot be evaluat-

ed. In the meat meal from Debrecen the feather content is highly apparent and according to our measurements is equivalent to 5.5–6.0% feather meal in the meat meal. The protein fraction analysis of feather meal manufactured on traditional desinfector shows a somewhat lower peak *A* than in feather (about 95%), ratios *B* : *C* and *D* : *E* are unchanged. The well evaluable peak *F* is the same as measured in feather. Peak *G* is minimal, practically not evaluable. Protein fraction analysis from the improved hydrolysed feather meal was not successful. In the chromatogram the amount of free amino acids covered the other fractions perhaps present. When we admixed to the meat meal from Tököl 3% feather meal manufactured on the traditional desinfector in 4 out of 5 determinations — on the basis of the indices described above — we recovered 2.5–3.2%, while in one case we found less than 2%. On adding 5% feather meal to meat meal 5 analyses gave results between 4.2 and 6.3%.

In summing up the results feather and feather meal contained 5–12 times more cysteine, 2.5–3 times more serine than meat meal. In contrast, meat contained 2 to 7 times more methionine and lysine and 2 to 4 times more histidine than feather and feather meal. On the basis of the cysteine content 3—4% feather or feather meal can be detected. The safety of detection could be improved by using the Cys × Ser per Lys × His × Met index. The use of this index shows the presence of 3% feather and the difference is significant at the $P \leq 1\%$ level.

The protein fraction analysis of feather and feather meal and meat and meat meal has shown peaks which were found only either in feather meal (peak *F*) or only in meat meal (peak *G*); peak *A* was found to be 15 times larger in feather meal than in meat meal and the ratio of some peaks differs significantly (peaks *B* and *G* and peaks *D* and *E*). Using these peaks or indices formed of them ($A \times B \times F$ per $C \times E \times G$) permits the detection of 1–2% feather meal admixed to meat meal. Protein fraction analysis cannot be used with chemically hydrolyzed feather meal.

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COCKTAILS PREPARED FROM ENZYMATICALLY SOLUBILIZED VEGETABLES AND FRUITS

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Consumption of fibrous vegetable juices, containing natural nutrients, vitamins and minerals, might be efficient means of the preservation of health.

Three vegetable cocktails (red beet–horseradish–paprika; celery–horseradish; tomato–paprika–celery–carrot) and two vegetable-fruit cocktails (apple–carrot–apricot; squash–orange) were prepared from vegetable and fruit nectars, produced by macerating enzyme. Chemical composition of the cocktails were the following (in g per dm^3): protein 1.4–2.4, carbohydrate 36–110, glucose 8–23, fructose 9–36, vitamin C 0.11–0.58 and β-carotene 0.12–0.30. The highest value of total pectin was measured in the celery and carrot cocktails (12.6 and 8.9 g per dm^3, resp.). Mineral contents of the cocktails varied as follows (g per dm^3): Mg^{++} 0.05–0.12, Ca^{++} 0.12–0.34, K^{+} 1.53–3.22, Fe^{++} 0.001–0.008. The lowest ratio of Ca–Mg was found in the red beet and tomato cocktails (1.12 and 1.57, respectively).

Keywords: vegetable cocktails, fruit cocktails, macerating enzymes

There are a lot of diseases peculiar to civilized communities and many of them result from fibre-depleted diets. Diets of plant origin, containing dietary fibers (pectin, cellulose, lignin) and minerals proved to have beneficial effects on human metabolism. With fiber containing diets the development of many metabolic (ANDERSON & CHEN, 1979; WAHLQUIST et al., 1979), cardiovascular (MORRIS et al., 1977) and gastro-intestinal (BURKITT, 1973) illnesses can be prevented.

In Hungary the consumption of vegetables is much below the desired level. The consumption of vegetables could be increased with fibrous vegetable cocktails. Vegetable juices prepared by enzymatic solubilization of the raw materials, contain almost all the valuable components (nutrients, vitamins, minerals, pectins, etc.) of the original plant tissues (ZETELAKI-HORVÁTH & VAS, 1980).

To produce the above type of soft drinks, various vegetables and fruits were treated by a macerating enzyme.

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1. Materials and methods

1.1. Enzyme preparation

An endo-polygalacturonase (endo-PG) preparation (Phylendonase) was used (produced at the Phylaxia Veterinary Biologicals Ltd., Budapest). according to the technology of ZETELAKI-HORVÁTH et al., 1981. The enzyme activity ($SPA_{75}^{NA\text{-}p}$) was 300 dm^3 per h per cm^3, measured on Na-polypectate substrate (Serva Entwicklungslabor, Heidelberg).

1.2. Materials used

The following fruits and vegetables were treated by endo-PG: apples (var. Jonathan), paprika (var. Red Tomato Shaped), tomato (var. Peto Mec), horseradish (unidentified), squash (unidentified), carrots (var. Chantenay), red beet (var. Purple Orb) and celery (var. Imperator).

1.3. Enzyme treatment

Ground vegetables (3–5 mm particles) were treated with 0.7 cm^3 per 100 g Phylendonase, while apples and tomatoes with 0.05 cm^3 per 100 g for 1 h at 40 °C.

After enzyme treatment, they were strained through 0.3 mm sieves, heat treated at 80 °C for 15 min and stored at −15 °C until use.

1.4. Preparation of cocktails

Juices obtained after enzyme treatment were mixed in different ratios and tested by a panel. Red beet, celery, squash, tomato and apple cocktails, prepared in this work, were found to be the best among 56 mixtures.

The composition of the cocktails was the following:

1. Red beet – red tomato shaped paprika – horseradish (55 : 25 : 20);
2. Celery–horseradish (90 : 10);
3. Apple–carrot–apricot (50 : 35 : 15);
4. Squash–orange (92 : 8);
5. Tomato–red tomato shaped paprika–celery–carrot (65 : 10 : 10 : 15).

Fibrous vegetable and fruit juices, prepared by enzyme treatment, were mixed in the desired ratio, homogenized, filled into bottles and pasteurized in closed state at 80 °C for 20 minutes.

1.5. Methods determining chemical composition

Protein content was determined from the ammonium ion content of the samples, after Kjeldahl digestion, using an automatic analyser (Contiflo, Labor MIM, Budapest).

Carbohydrate content of the samples was determined by the method of HERBERT and co-workers (1971).

Reducing sugar contents of the samples were determined by the NELSON (1944) method. Fructose content was measured by the method of SMITH and MCCURDY (1952) after incubation at 52 °C for 6–7 min in the Contiflo automatic analyser.

Pectin content of the samples was determined by the method of MCCOMB and MCCREADY (1952).

Cellulose and *lignin* determinations were carried out by the method of COLLINGS and co-workers (1978).

Vitamin C content was determined by thin layer chromatography according to PETRÓ-TURZA (1971) and β-carotene by the spectrophotometric method of KRAMER-FALUS (1971).

Minerals (Mg^{++}, Ca^{++}, K^{+} and Fe^{++}) were determined by atomic absorption spectrophotometry.

1.6. Organoleptic evaluation

Cocktails were tested by a panel of eight members. The final value of the cocktails was calculated from the mathematical mean of the scores given for taste, colour, consistency and the general impression of the samples.

2. Results

2.1. Chemical composition of the raw materials

Vegetables having high water and low sugar content belong to foods of low caloric value. Reducing sugars represent only a low concentration in the carbohydrate content of vegetables a considerable part of which being fructose.

In the course of enzymatic decomposition of vegetables, not only the nutrients and vitamins but a great part of their pectin and cellulose content gets into the juices. So, in the course of the consumption of vegetable cocktails, the latter can exert their nutrition-physiological and health-preserving effect.

Vegetables are rich sources of vitamins and provitamins (vitamin B group, vitamin C and β-carothenes) and minerals (TARJÁN & LINDNER, 1972; ŠULC, 1984), the majority of which can be recovered during enzyme treatment (ZETELAKI-HORVÁTH & VAS, 1980). In this work about 59 – 88 % of the valuable nutrients, vitamins and minerals were recovered after enzymatic solubilization.

2.2. Preparation of cocktails

From the fibrous vegetable and fruit juices of fine dispersity (the majority of the particles belong to the size range of 5–50 μm) (ZETELAKI-HORVÁTH & URBÁNYI, 1978) cocktails were mixed. The components of the various cock-

tails were chosen so, that mixing would result in a new characteristic taste and a pleasant colour. The colour of the individual components should have an improving effect on one another.

The main aim of cocktail mixing was naturally the production of new composition of aroma, nutrients, vitamins and minerals, suiting the preference of the various strata of consumers (healthy, reconvalescent or ill people, children, old people, sportsmen, etc.).

2.2.1. Red beet cocktail. Red beet being a field plant and storable for a long period, is a good raw material for cocktail production with special regard to its intensive colour.

According to statistical data, it is effective in the preservation of health and in increasing resistance of human organisms to various diseases.

In the course of this work a cocktail of salty character was mixed from red beet juice and the juices of horseradish and red tomato shaped paprika.

2.2.2. Celery cocktail. Celery is also a field crop of high keeping quality, available on the market for 6–7 months. It can be almost completely decomposed by macerating enzymes, has a high pectin and fiber content and can be mixed with vegetables and fruits as well. In this work, celery juice was flavoured with horseradish and was given a salty character.

2.2.3. Apple cocktail. Apples, having a pleasant flavour and a high vitamin C content, are grown in large quantities in Hungary. Those which do not meet quality requirements can well be utilized as components of vegetable cocktails. Carrot is one of the most valuable vegetables, having high β-carotene, pectin and fiber contents. It can be grown in the field and it is also available throughout the year. Apricot has nice colour and it is one of the best flavoured fruits of Hungary. Because of its high price, it can be used only in small quantities as a flavouring component of cocktails.

In our experiments apples were mixed with carrots and apricot and a nice fruit and vegetable cocktail was obtained of sweetish character.

2.2.4. Squash cocktail. Among the vegetable juices tested squash had the most pleasant colour. It is a field plant with high β-carothene content. It is easy to grow and cheap. It is available in the autumn and winter seasons. Squash is suitable for the production of cocktails of sweet character. In this work orange concentrate was used as flavouring material to the squash juice.

2.2.5. Tomato cocktail. Tomatoes are grown in large quantities in Hungary. Tomato concentrates are produced in the canning factories in the season and are available throughout the year. Tomato can be mixed with other vegetables the flavour of which is harmonizing with each other. The resulting cocktail is also of salty character. It can be consumed instead of soup, or as an appetizer, as source of vitamins and minerals or as salt supplement. In the case of the present cocktail, tomato juice was mixed with the juices of tomato shaped paprika, celery and carrots.

2.3. Chemical composition of the cocktails

2.3.1. Protein, carbohydrate and vitamin content. Dry matter content of the cocktails varied between the range of 65–94 g dm^{-3}. There were not great differences in the protein contents of the produced cocktails (1.4–2.4 g dm^{-3}) but their carbohydrate contents were rather different. Low carbohydrate content was found in the tomato, red beet and celery cocktails (36.53 and 55 g dm^{-3}, resp.) while much higher in the apple and the highest in the squash cocktails (80 and 110 g dm^{-3}).

Concentration of the reducing sugars was also the highest in the apple and squash cocktails (57 and 54 g dm^{-3}), respectively. The ratio of fructose to glucose proved to be the most favourable in the tomato and celery cocktails (3.1 and 3.4, resp.) as shown in Fig. 1.

The highest vitamin C content has been found in the apple cocktail, followed in decreasing sequence by the squash, tomato and red beet cocktails (0.47, 0.44 and 0.11 g dm^{-3}, resp.). The β-carothene content of the prepared cocktails varied between 0.12–0.30 g dm^{-3}.

All the five cocktails belong to the category of low caloric products, their energy content varied in the range of 520–750 kJ dm^{-3} (the only exception was the squash cocktail with an energy content of 1330 kJ dm^{-3}).

2.3.2. Pectin, cellulose and lignin content. The mass of fibers separated from the cocktails by centrifugation was the highest in the apple cocktail

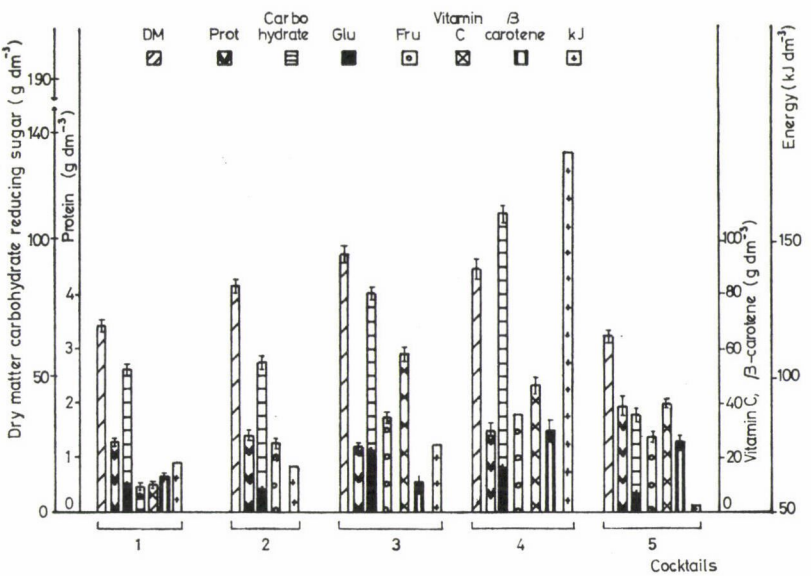

Fig. 1. Chemical composition of vegetable and vegetable–fruit cocktails. 1: Red beet; 2: celery; 3: apple; 4: squash; 5: tomato

(43.5 g dm^{-3}). It was followed in decreasing sequence by the squash, tomato, celery and red beet cocktails.

Considering the pectin content of the vegetables, a very highly significantly highest pectin content was found in the serum of the celery cocktail (11.27 g dm^{-3}) while the next highest pectin concentration (6.61 g dm^{-3}) was detected in the apple cocktail (Fig. 2).

The highest pectin content of the fibre fraction was found in the apple cocktail (2.25 g dm^{-3}) from which that of the tomato cocktail did not differ significantly.

Cellulose content proved to be the highest in the squash cocktail, which was very highly significantly higher than those in the four other cocktails.

Examining the pectin constituents of the fiber fractions of the cocktails, water soluble pectins were found in the highest proportion in the celery, apple and tomato cocktails (59, 58 and 44 mg per 100 g, resp.) while in the squash cocktail the ratio of pectates and pectinates (soluble in oxalate) was higher (44 mg per 100 g). Protopectins, soluble in NaOH were found in the highest concentration in the tomato cocktail (22 mg per 100 g) as shown in Fig. 3.

The fiber fraction of the red beet cocktail was not sufficient for the analysis of pectin.

2.3.3. Concentration of minerals. Minerals have an important role in the human organism. They are components of different enzymes, vitamins, body fluids and the skeleton. They ensure the ion balance of the organism and the osmotic pressure of the cells, they play a role in blood coagulation and participate in neuromuscular conduction.

In the five cocktails prepared, the concentration of Mg^{++}, Ca^{++}, K^{+} and Fe^{++} was determined. The highest Mg content was found in the red beet cock-

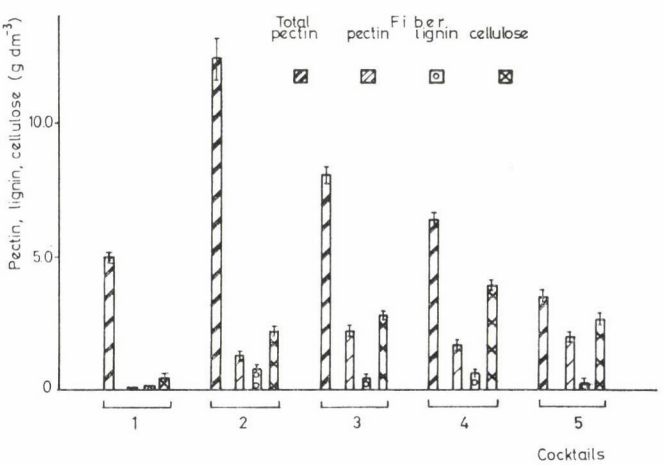

Fig. 2. Pectin, cellulose and lignin content of the cocktails. For symbols see Fig. 1

tail (0.125 g dm^{-3}), this was followed in decreasing order by the celery and tomato cocktails (Fig. 4).

The highest Ca content was detected in the celery and the squash cocktails (0.343 and 0.279 g dm^{-3}) while in the red beet and apple cocktails a very highly significantly lower Ca content (0.139 and 0.125 g dm^{-3}) was measured.

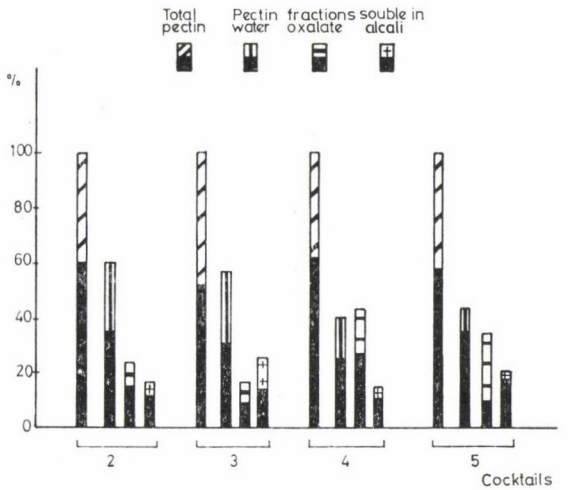

Fig. 3. Pectin constituents of the fiber fraction of the cocktails. For symbols see Fig. 1

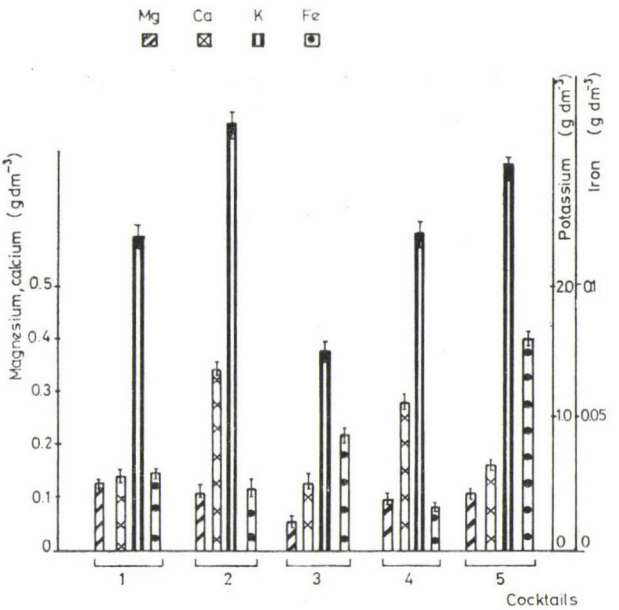

Fig. 4. Concentration of several minerals in the cocktails. For symbols see Fig. 1

A rather high K concentration was found in the celery and tomato cocktails (3.22 and 2.85 g dm^{-3}, resp.) while in the apple cocktail only 1.53 g dm^{-3} could be detected.

Iron was found in the highest concentration in the tomato cocktail (0.008 g dm^{-3}) and the lowest (0.0015 g dm^{-3}) in the squash cocktail.

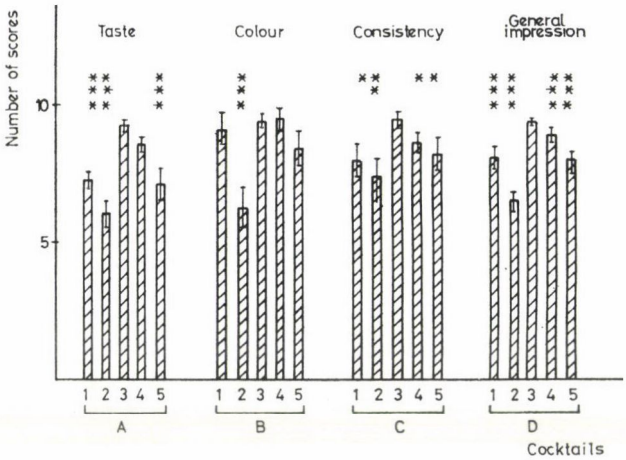

Fig. 5. Results of the organoleptic tests of the cocktails. * Significant at $P \leq 0.05$ probability level; ** highly significant at $P \leq 0.01$ probability level; *** very highly significant at $P \leq 0.001$ probability level.

2.3.4. Organoleptic testing of the cocktails. In the course of the organoleptic evaluation of the cocktails, consisting of several components, the odour of the samples could not be taken into consideration, having no special basis of comparison.

According to the opinion of the panel of eight persons, the taste of the apple and squash cocktails proved to be very good, significantly the best (Fig. 5).

From the point of view of colour, the squash cocktail proved to be the most pleasant, but the scores given by the panel did not differ significantly from those of the red beet, apple and tomato cocktails, respectively. The lowest scores were given to the celery cocktail, having no characteristic colour.

The consistency was found to be the best in the case of apple cocktail. It proved to be significantly better than those of the squash, tomato and red beet cocktails and very highly significantly higher than that of the celery cocktail. The latter was found to be a little thick.

From the point of view of general impression the apple cocktail proved to be very highly significantly the best.

3. Conclusions

Enzymatic solubilization of vegetables and fruits result in not only a lower loss in the raw materials, nutrients, vitamins and minerals (ZETELAKI-HORVÁTH & GÁTAI, 1977; ZETELAKI-HORVÁTH et al., 1985) but a higher fiber content of the product with special respect to the pectin content as well. Pectin is a valuable component of the dietary fiber in vegetables and fruits (HORVÁTH-MOSONYI et al., 1981; RIGÓ et al., 1982) and plays an important beneficial biological and nutrition physiological role. By causing a feeling of saturation and establishing a negative energy balance (by increasing the faeces weight) fibers are effective against obesity. Water adsorption, cation exchange capacity, swelling and gelformation are all physical characteristics of the polysaccharide constituents of plant fibers (EASTWOOD, 1973). Pectins are able to adsorb bile acids and decrease the cholesterol level of the blood (SPILLER & AMEN, 1975; ANDERSON & CHEN, 1979). Fibers control the concentration of the electrolytes in the organism, influence the transit time of food through the intestine (CORINALDESI et al., 1982) and they have a detoxicating role by the adsorption of bi- and polyvalent ions (ROBERTSON et al., 1980).

The mixtures of the nutrients and vitamins of different origin are more advantageous from the point of view of nutrition physiology (TARJÁN, 1975) and health preservation, as well.

The effect of pectins differs according to the degree of esterification and according to their origin. While apple pectin can control the cholesterol level of the blood, carrot pectin plays an important role in the detoxication of bi- and polyvalent ions (BOCK & KRAUSE, 1978).

The vegetable cocktails proposed in this work can be used as low caloric foods and some of them are suitable for diabetic diet, too. The red beet and the tomato cocktails with their low Ca–Mg ratio improve the ion balance in the organism, for in Hungary, the concentration of Ca in the diets is often high.

The cocktails of salty character can be used as salt supplements for sportsman or to ill people who need K and Mg supplementation. The utilization of these minerals is much better in their natural form.

The fact that cocktails, being more or less sweet in character, proved to be the best by the panel, is the consequence of old traditions. Namely, consumers have been accustomed to a large variety of sweet juices on the market for several decades.

The consumption of the cocktails made from enzymatically solubilized vegetables and fruits exerts its health preserving effect through its fiber, pectin, vitamin and mineral content and low Ca–Mg ratio.

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Acta Alimentaria, Vol. 15 (2), pp. 163—164 (1986)

BOOK REVIEWS

Sorption isotherms and water activity of food materials

W. WOLF, W. E. L. SPIESS and G. JUNG (Eds.)

Science and Technology Publishers Ltd., Hornchurch, Essex, England; 236 pages

The book is a bibliographical compilation on the subject of sorption isotherms and water activity in food materials, published by the three authors mentioned above. The three authors also published their own results in studying sorption isotherms of food materials in the publication list.

The bibliography now comprises 2201 articles dealing with the theoretical and practical aspects of sorption isotherms as well as their measuring data in food materials. The individual references were classified into 5 subject groups and each reference was supplemented by a code number as below:

1. General descriptions
2. Thermodynamics of the sorption process
3. Measuring methods
4. Influence of water activity on product stability
5. Sorption data

Five thematical tables were also arranged according to the code numbers of subjects and the consecutive numbers of references indicated. So the references of the individual subjects can be easily found.

The value and utility of the bibliography is greatly increased by a collection of sorption data literature. Compiled in these tables, there are 600 literature sources related to sorption isotherms or water activity in food materials, containing the serial number of the authors as well. So the relevant literature source containing the sorption data of a given food material is easily accessible to everybody interested in the data of a certain subject matter.

Authors give information on the accessibility of the original literature as well.

L. SZALAI

Microbial associations and interactions in food

Proceedings of the 12th International IUMS—ICFMH Symposium Budapest, Hungary 12–15 July, 1983

I. KISS, T. DEÁK and K. INCZE (Eds.)

Akadémiai Kiadó, Budapest, Hungary and D. Reidel Publishing Corp., Dordrecht, Holland, 1985; 470 pages

Academician Holló gave, in his address of welcome, a comprehensive idea of food science and food industry in Hungary to-day. The organizers of the Symposium commemorated the late Dr. Károly Vas, academician, who was well known in scientific circles abroad, too, and passed away recently.

Akadémiai Kiadó, Budapest
D. Reideb, Dordrecht

The publication of 470 pages contains the complete papers of 46 foreign and 20 Hungarian participants. Four papers were presented to the plenary session:

MOSSEL, D. A. A.: Half a century of microbial ecology of foods;

DEÁK, T.: Microbial-ecological principles in controlled atmosphere storage of fruits and vegetables;

HOBBS, B. D.: Intestinal pathogens and the environment;

SKOVGAARD, N.: Vegetables as an ecological environment for microbes.

Further papers read at the symposium can be listed in one of the following six groups:

1. Pathogenic microorganisms. 16 papers dealt, among others, with Salmonella contamination in milk, in egg powder and meat products and with the steadily increasing importance of pathogenic microorganisms.

2. Methods. The subject was treated in 15 papers. The major part of the papers discussed problems related to the application of selective media, to sampling, to rapid methods of analysis, to gas-chromatographic analysis and identification and to the numerical characterization.

3. Spoilage microorganisms. In the six papers belonging to this group the microorganisms playing a role in the spoilage of different foodstuffs (bread, non-alcoholic drinks, meat products, sweets) were studied.

4. Miscellaneous. In this group 4 papers were read treating the following subjects: toxin, aflatoxin, cellulase enzyme and hygienic problems of slaughterhouses.

5. Useful microorganisms. Six papers were presented in this section. The subjects of the papers were: starter cultures of bread, meat and milk products and interaction of the microflora and finally the composition of brewing yeast.

6. Preservation. In this section 15 papers treated classical and modern methods of preservation. Some of the papers discuss the cold storage of meat and cold combined with treatment under vacuum, the treatment of meat products and black pepper with gamma radiation or gamma radiation combined with either ethylene oxide or NaCl. In other papers the effect of CO_2, and of various preservative agents (oleoresinous spices, pimaricin, sage and inhibitors) on the storage life of meat, or on the viability and growth of certain microorganisms, was investigated.

The authors of the papers presented the latest results of their experiments in the field of food microbiology. The papers contained the description of a great number of methods utilizable directly in food research. Thus, the book is of interest not only to the theoreticians but for the man of practice, too. The proceedings of the Symposium and the papers presented there are a worthy continuation of the earlier Symposiums and publications.

L. NYESTE

RECENTLY ACCEPTED PAPERS

Viscosity behaviour of *Vicia faba* protein isolates and their acetylated derivatives
SCHMIDT, G., SCHMANDKE, H. & SCHÖTTEL, R.

Determination of phylloquinone (vitamin K1) in raw and processed vegetables using reversed phase HPLC with electrofluorometric detection
LANGENBERG, J. P., TJADEN, U. R., DE VOGEL, E. M. & LANGERAK, D. IS.

Investigations into some enzyme activities of Finnish wheats grown in Hungary
PÁRKÁNY-GYÁRFÁS, A., VÁMOS-VIGYÁZÓ, L., SALOVAARA, H. & KOIVISTOINEN, P.

Disinfestation of commercially packed dates by a combination treatment
AHMED, M. S. H., HAMEED, A. A. & KADHUM, A. A.

Correlation between sensory and gas-chromatographic measurements on grapefruit juice volatiles
PINO, J., TORRICELLA, R. & ÖRSI, F.

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Title. The title should be concise and informative. It must be followed by the authors' names and the address(es) of the institute(s) where the work was carried out.

Summary. The article should be preceded by a summary (not exceeding 150 words) giving a self-explanatory compendium of the essence of the paper. At the end of the summary some 4 keywords should be put in alphabetical order.

Text. The article should be divided into the following parts: *Introduction* beginning on a new page without a title containing a survey of related literature and the objectives of the work, followed by *Materials and methods; Results; Conclusions.*

Symbols. Units, symbols and abbreviations should be used according to SI (System International) rules. Unusual abbreviations must be explained in the text or in an appendix.

Figures and tables. Illustrations must be cited and numbered in the order they appear in the text. All line drawings should be submitted as clear, glossy, black and white originals or photographs of good quality. The author's name and the title of the paper together with the serial number of the figure should be written on the back of each figure. Figures and Tables, each bearing a title, should be self-explanatory and numbered consecutively. The number of measurements (n), their mean values ($\bar{x}$) and standard deviations ($\pm s$) should be indicated. To prove the objectivity of conclusions drawn from the results mathematical statistical methods must be used. Quantitative evaluation of data is indispensable.

References. The reference list (Literature) should be in alphabetical order as follows:

Periodicals: Names and initials of all the authors; year of publication in parentheses; colon; title of the paper; title of the periodical; inclusive page numbers.

Books: Names and initials of all the authors; the year of publication in parentheses; colon; title of the book; publishing firm, place of publication; inclusive page numbers.

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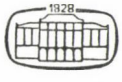

AKADÉMIAI KIADÓ
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FRACTIONATION AND CHARACTERIZATION OF *PISUM SATIVUM* GLOBULINS

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Study of the effect of pH on the solubility of the globulins extracted from *Pisum sativum* var. Lincoln showed that the minimum solubility was at pH 3.5. The total globulins were separated into two main components (legumin and vicilin) by chromatography on DEAE-cellulose and Sephacryl S-200 column. Gel filtration chromatography on calibrated Sephacryl S-200 column indicated that the legumin has a molecular mass of 300 000–400 000 whereas a value of 135 000 was determined for vicilin.

The spectrophotometric studies showed that legumin was free of nucleic acid whereas vicilin was contaminated or conjugated with other compounds. Electrophoretic examinations revealed the presence of two major bands on the polyacrylamid gel electrophoretograms for total globulins.

The amino acid composition of total globulins and legumin separated from Sephacryl S-200 column indicated a great glutamic acid content and to a lesser extent arginine and lysine. All fractions were poor in sulfur containing amino acids.

Keywords: pea, pea globulin, amino acid composition

Leguminous seeds are an important source of protein in the human diet. So it is understandable that they have received a great deal of attention. An early extensive study of chemical, nutritional and functional properties of the legume crops has been undertaken by OSBORNE and CAMPBELL (1898). They showed that much of the protein of legume seeds was salt soluble globulin, and they were able to separate this fraction from *Pisum sativum* into two components, legumin and vicilin. The same two globulin components were also separated from pea seeds by chromatography on DEAE-cellulose (GRANT & LAWRENCE, 1964).

The pea seed globulin components were also studied by many investigators using two dimensional gel electrophoresis, immunoaffinity chromatography, zonal isoelectric precipitation and DEAE-cellulose chromatography (KRISHNA et al., 1979; CASEY, 1979; CASEY & SANGER, 1980).

The work reported here was undertaken to extract and characterize the globulins of *Pisum sativum* variety Lincoln cultivated in Egypt. The results obtained are presented in this paper.

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1. Materials and methods

1.1. Preparation of dry defatted meal

The dehulled *Pisum sativum* seeds were ground in a coffee grinder to a fine flour, then blended with ice cold acetone. The defatted material was air-dried over-night at room temperature and stored in a closed container at 4 °C until required.

1.2. Extraction of pea globulins

The fat free meal was extracted twice with distilled water, using a ratio of 1 : 20 (w/v) for meal to water. The suspension was shaken for 2 h at 4 °C and then centrifuged at 5000 r.p.m. for 30 minutes.

The insoluble material was washed twice with distilled water and extracted twice with 1 mol dm^{-3} NaCl solution. After centrifugation, the supernatants were combined and dialyzed several times against distilled water for 72 h at 4 °C (10×2 dm^3). The precipitate was collected by centrifugation, washed twice with distilled water, combined with the precipitate formed from water extractable protein after dialysis and then freeze-dried.

1.3. Determination of solubility

Protein solutions (1 mg cm^{-3}) were prepared by dissolving the freeze-dried protein in 0.5 mol dm^{-3} NaCl solution. Aliquots of 15 cm^3 were adjusted to the desired pH by adding either 5 mol dm^{-3} HCl or NaOH and then stirred for 15 minutes at room temperature. Turbidity of protein solutions at various pH values was measured at 600 nm. After leaving the protein solution overnight at 4 °C, it was centrifuged at 10 000 r.p.m. for 30 minutes. Protein concentration in the supernatant was measured by recording the absorbance at 280 nm.

1.4. Fractionation of pea globulins on DEAE-cellulose column

A preswollen microgranular form of DEAE-cellulose (DE 52; column: 1.5 m, i.d. 4 mm) was equilibrated according to the method recommended by the manufacturer's instructions. The required amount of exchanger was equilibrated with 30 mmol phosphate buffer with pH 8.0 and containing 0.15 mol dm^{-3} NaCl.

The protein solution in the starting buffer was applied on the column via a three way tap. Gradient elution was applied using stepwise increase of NaCl concentration (0.15; 0.30; 0.45 and 0.60 mol dm^{-3}).

Fractions of 10 cm^3 were collected at a flow rate of 0.5 cm^3 per minute. The absorbance at 280 nm was measured for each fraction.

1.5. Fractionation of pea globulins by gel filtration

Sephacryl S-200 equilibrated in 0.1 mol dm^{-3} TRIS-HCl pH 7.6 containing 0.5 mol dm^{-3} NaCl was packed into a column (2.5 × 100 cm). The protein sample was then applied to the column from a suitable reservoir via a three way tap. The height of the buffer reservoir was adjusted to give a flow rate of about 20 cm^3 per h and fractions of a suitable size were collected using an LKB Vectorac 7000 Fraction Collector (LKB, Bromma I, Stockholm, Sweden).

The optical density of each fraction was monitored at 280 nm. A portion of each fraction was analyzed by the Lowry method (Folin phenol reagent) at 750 nm (LOWRY et al., 1951).

For the determination of molecular mass of the components to be separated the column was calibrated with proteins of known molecular mass.

1.6. Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis of protein fractions was performed on 7.5% (w/v) polyacrylamide gels. The method used was disc electrophoresis as described by DAVIS (1964). The gels were examined for protein bands by spectrophotometric scanning or by the use of protein specific stains, such as Amido Black or 0.2% Coomassie Brilliant Blue.

The excess of stain was removed by repeated washing with 7% (w/v) aqueous acetic acid if Amido Black was used (NAGAI et al., 1964) for staining. Glacial acetic acid : ethanol : water (2 : 5 : 13 by volume) as destaining solution was used for Coomassie Brilliant Blue until the bands were distinctly dark blue or deep blue, respectively, on a colourless background. The gels stained with Amido Black could be scanned at 600 nm and those stained with Coomassie Brilliant Blue at 609 nm on a Gilford 2400-S spectrophotometer, fitted with the 2410-S linear transport attachment, to record the positions of the protein bands.

1.7. Determination of amino acids for protein fractions

Appropriate amounts of the total pea seed globulins and their purified fractions obtained by fractionation on Sephacryl S-200 column, were hydrolyzed in sealed tubes at 110 °C for 24 hours with 6 mol dm^{-3} HCl. The excess HCl was then evaporated under vacuum, with occasional addition of distilled water 3–5 times. The residue was dissolved in 10% isopropanol (v/v) then filtered and the final volume was made up to 5 cm^3 in a measuring flask with the same solvent. The percentage of each amino acid was determined by an automatic amino acid analyser (Mikrotechna Praha, AAA 881), according to LÁSZTITY and TÖRLEY (1982).

2. Results and discussion

2.1. Effect of pH on the solubility

The results of the effect of pH on the solubility of globulins extracted from *Pisum sativum* variety Lincoln are shown in Fig. 1. The minimum solubility of the globulins, as shown in Fig. 1, was at pH 3.5. The increase in the solubility of pea globulins at pH below 2 could be attributed to protein denaturation. At the isoelectric point (pH 3.5) about 30% of the pea globulins were still so-

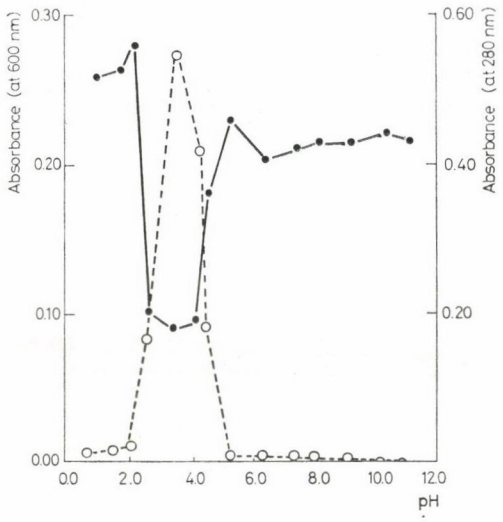

Fig. 1. Effect of pH on the solubility of pea globulins. o----o: 600 nm; • — • : 280 nm

luble, this may be due to the composition of the proteins since some proteins, albumins and globulins may not precipitate at their isoelectric pH (SEFA-DEDEH & STANLEY, 1979).

2.2. Fractionation of total globulins by ion exchange chromatography

A portion (150 mg) of lyophilized globulins was dissolved in 10 cm^3 buffer, and applied to the top of DEAE-cellulose column equilibrated with the same buffer.

The elution profile (Fig. 2) showed the separation of the globulins into two major components referred to as *A* and *B*. The first component eluted from DEAE-cellulose column (peak *A*) is assumed to correspond to vicilin and the second to legumin as indicated by GRANT and LAWRENCE (1964). The minor component (peak *C*), in Fig. 2 did not appear consistently and could be legumin dimer. Our results agree with those obtained by other workers (GRANT &

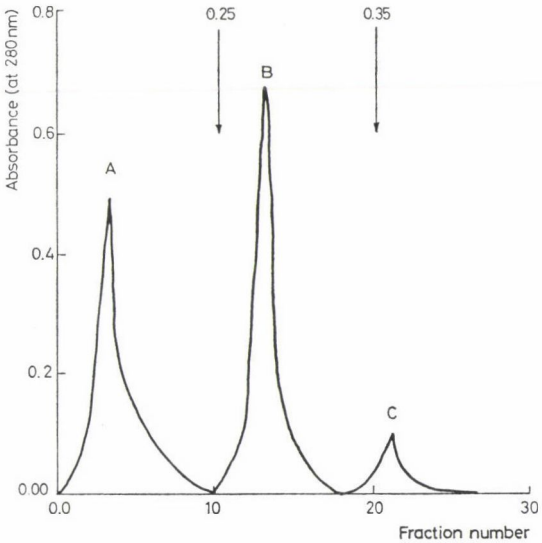

Fig. 2. Chromatography of total globulins from peas on DEAE-cellulose column. The molarity of buffer was 0.25 and 0.35, respectively

LAWRENCE, 1964). The ratio of absorbance at 280 nm to that at 260 nm (A_{280}/A_{260}) was calculated to be 1.06, 1.42 and 1.38 for components A, B and C, respectively, and this indicated the contamination of peak A with nucleic acids (WARBURG & CHRISTIAN, 1942).

2.3. Fractionation of total globulins by gel filtration

The elution profile for a typical experiment is shown in Fig. 3. The protein in the first peak emerging from the column was eluted in the void volume, which indicated a protein of larger molecular size than that eluted in the second peak.

Using repeated precipitation by dilution or heat treatment, OSBORNE and CAMPBELL (1898) were able to separate *Pisum sativum* globulins into two major components, legumin and vicilin.

The legumin has a sedimentation coefficient of approximately 11 S and a molecular mass in the region of 300 000–400 000, while vicilin is a smaller protein with sedimentation coefficient of 7 S and a molecular mass around 140 000 (DERBYSHIRE et al., 1976). Thus it could be concluded from the elution profile shown in Fig. 3 that the first component (peak A) corresponds to legumin while the second peak B contains vicilin. The elution volume for the second component (peak B) indicated that the molecular mass of this component is approximately 135 000 which agrees with the value reported in the literature (DERBYSHIRE et al., 1976). When absorbance was measured at 260 nm

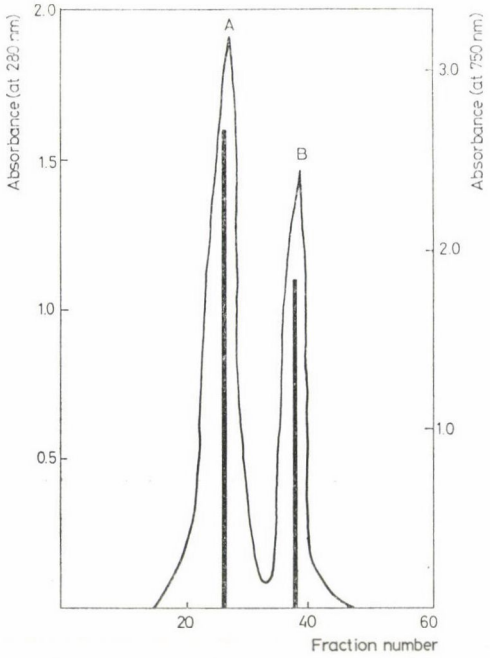

Fig. 3. Chromatography of total globulins from peas on Sephacryl S-200 column. ——: A_{280}; ▬▬: A_{750}

as well as at 280 nm for the fractions eluted from the Sephacryl S-200 column, it showed that the second peak (vicilin) contained protein with higher absorbance at 260 nm than at 280 nm which suggested the presence of nucleic acids. Therefore, protein assays by the Lowry procedure (Lowry et al., 1951) were performed on selected fractions and the results indicated that peak *A* contained more protein than peak *B*.

The molecular size of the pea globulins has always been indicative of the existence of subunit structure, this has been substantiated by data from dissociation studies and N-terminal amino acid analysis. The legumin and vicilin split into subunits which could be separated by electrophoresis. The subunits of pea legumin were separated by two dimensional gel electrophoresis into at least 5 acidic (molecular mass about 40 000) and 5 basic (molecular mass 20 000) subunits (Krishna et al., 1979). Furthermore, the subunit of vicilin of both *Vicia faba* and *Pisum sativum* show qualitative changes during seed development (Wright & Boulter, 1972). This is an indication of the heterogeneity of this protein.

The ultraviolet absorption spectra of the isolated globulins are presented in Fig. 4. The legumin (peak *A* in Fig. 3) showed a spectrum similar to those of most proteins and exhibited maximum at 280 nm and minimum at 251 nm.

The ratio of absorbance at 280 nm to 260 nm was 1.5 indicating that this component was free of nucleic acid. On the other hand, the ultraviolet absorption spectrum for vicilin fraction (Fig. 4) was characterized by a maximum at 265 nm and minimum at 248 nm and ratio of absorbance at 280 nm to 260 nm was estimated to be 0.92. These results point to the presence of nucleic acids or other compounds contaminating or conjugated with the protein molecule e.g. carbohydrate as has been reported in vicilin separated from *Vicia faba* (BAILY & BOULTER 1972), *Phaseolus mungs* (CHAVAN & DJURTOFT, 1982).

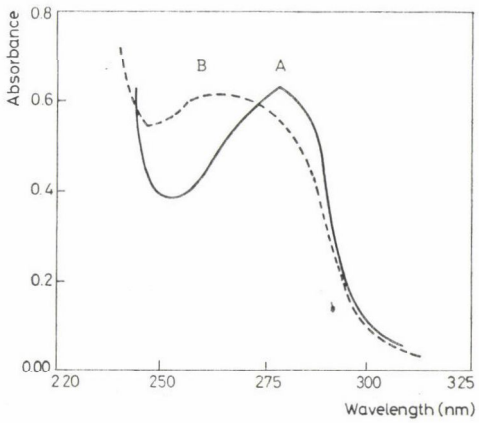

Fig. 4. Ultraviolet absorption spectra of globulin components of peas obtained from Sephacryl S-200 column.

The latter preparation contained 12.8% carbohydrate while the vicilin prepared from *Vicia faba* contained less than 2% neutral sugars and 0.2% hexosamine.

The total pea globulins were subjected to electrophoresis on polyacrylamide gels as the supporting medium. Figure 5 shows the results of this experiment. It can be noted that the total globulins consisted of two major components of low and medium electrophoretic mobilities (Fig. 5). Similar electrophoresis of vicilin and legumin preparations revealed that the slow migrating band was vicilin and the faster band was legumin (JACKSON et al., 1969).

Examination of samples taken from the loading edge of the first peak eluted from the Sephacryl S-200 column by polyacrylamide gel electrophoresis (Fig. 3) indicated that this peak contained only legumin (Fig. 5). On the other hand, electrophoretic examination of samples taken from the trailing edge of the same peak showed slight contamination with vicilin.

Peak *B* resolved by fractionation on Sephacryl S-200 column (Fig. 3) contained mainly vicilin with trace amount of legumin as revealed by polyacrylamide gel electrophoresis.

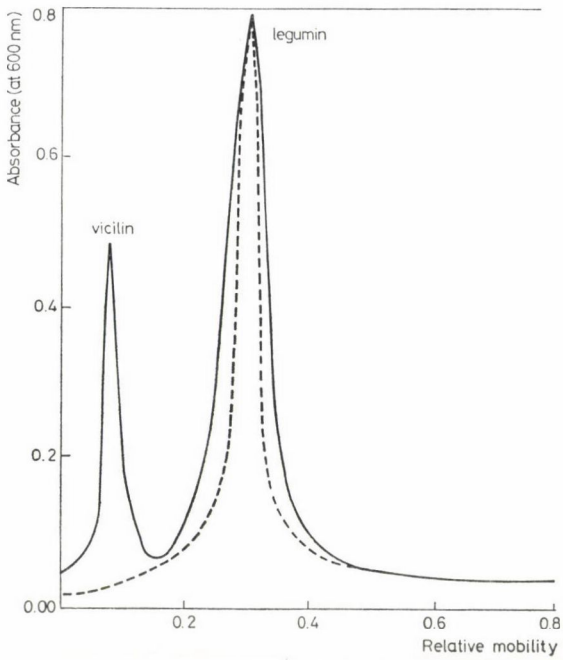

Fig. 5. Densitometric scanning of polyacrylamide gel electrophoretic patterns of pea globulins. ——: total globulins; – – –: purified legumin

2.4. Amino acid composition of pea globulins

The amino acid composition of total globulins and legumin separated from Sephacryl S-200 column as well as the value reported by other workers for *Pisum sativum* globulins are presented in Table 1. It is evident from Table 1, that glutamic acid is the major component; lesser components are the basic amino acids, arginine and lysine. The amino acid analysis (Table 1) also indicates that legumin contains less threonine, cystine and methionine than found in total globulins.

When comparing the amino acid composition of legumin separated in the work reported here with those of BOULTER and DERBYSHIRE (1971), we observed that our legumin contained higher amounts of tyrosine, serine, alanine and aspartic acid but lower levels of cystine and methionine.

Apart from the sulphur containing amino acids (methionine and cystine), valine and threonine, the *Pisum sativum* globulins (Table 1) contained ample amounts of the essential amino acids (FAO/WHO, 1973). The use of legume seeds in the preparation of balanced protein diets is limited by their generally low levels of the sulphur amino acids, methionine and cystine. The latter can partly replace the dietary requirements for methionine (JOHNSON & LAY, 1974).

Table 1

Amino acid composition of pea globulin
(g amino acid per 100 g protein)

Amino acid	Total globulin	Legumin	Total[a] globulin	Legumin[b]
Alanine	3.42	4.67	4.2	3.7
Arginine	8.14	9.12	10.0	10.5
Aspartic acid	13.25	13.70	14.0	12.5
Cystine	0.60	0.33	—	0.71
Glutamic acid	18.44	19.50	24.9	21.1
Glycine	3.70	3.90	4.1	3.4
Histidine	2.30	2.10	2.6	2.8
Isoleucine	4.20	5.10	3.8	2.6
Leucine	8.17	6.35	5.5	5.5
Lysine	9.11	4.81	7.9	4.9
Methionine	0.95	0.26	0.8	0.65
Phenylalanine	6.30	4.33	5.7	4.9
Proline	3.80	4.2	4.5	4.3
Serine	6.11	5.62	6.1	4.5
Threonine	3.20	2.11	3.6	2.9
Tryptophan	0.75	—	0.9	1.06
Tyrosine	6.55	4.22	3.6	3.3
Valine	3.80	4.40	4.6	4.6

[a] BHATTY (1982).
[b] BOULTER and DERBYSHIRE (1971).

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VISCOSITY BEHAVIOUR OF *VICIA FABA* PROTEIN ISOLATES AND THEIR ACETYLATED DERIVATIVES

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Neutral aqueous suspensions of *Vicia faba* protein isolates and their acetylated derivatives differ in their viscosity behaviour depending on the concentration as well as on the temperature.

The order of magnitude of viscosity changes conditioned by a thermal treatment of the protein suspensions depends clearly on the starting viscosity.

Suspensions of acetylated *Vicia faba* protein isolates show a thermally conditioned viscosity change at a temperature which lies about 20 °C lower and with shorter times of thermal influence, compared with suspensions of *Vicia faba* protein isolates.

The suspensions of the studied protein isolates exhibit mostly plastic flow, only low viscous solutions show pseudoplastic flow. The plastic percentage grows with increasing protein concentration, with increasing heating temperature, and with increasing time of heat action. The used method for the utilization of flow diagrams enables the perception and the supervision of changes in the flow properties in dependence on protein concentration and influences of temperature.

Keywords: protein isolates, *Vicia faba* protein, protein viscosity

Viscosity is a frequent and often a critical parameter of liquid and semiliquid media. Its measurement and its description is always difficult when the product exhibits complex rheological properties (SHETH, 1976). The majority of food items can not be described by rheological basic models as there are Newtonian, Bingham, dilatant, plastic and pseudoplastic flow, respectively, but they exhibit normally a mixture of different flow properties (SHETH, 1976; KLETTNER, 1973). Therefore, the present paper will be restricted to the elaboration of key values of viscosity with reference to border conditions, especially to the past history of the sample production. In addition to that, flow curves were registered in order to find out quantitatively the predominant established flow model. Sections of the flow curve were exploited mathematically according to Ostwald-de Waele. The aim of the present study was to detect the influence of protein concentration and particularly the influence of a constant temperature in dependence on their exposure time as well as the action of different temperatures at a constant exposure time on the viscosity behaviour of suspensions of *Vicia faba* protein isolates. In addition to that a cyclic temperature test at a constant rate of shear, modified according to UMEYA and co-workers (1981) was carried out.

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1. Materials and methods

1.1. Materials investigated

Product A: *Vicia faba* protein isolate (VfI), prepared in the laboratory, N content: 14, 7%.
Product B: VfI, prepared in the small-scale pilot plant, N content: 12.9%.
Product C: Acetylated VfI, prepared in the laboratory, N content: 13.7%, acetylation degree: 90% (blocked lysine).
Product D: Acetylated VfI, prepared in the small-scale pilot plant, N content: 13.1%, acetylation degree: 70% (blocked lysine).

These products were prepared from the same raw material in the following manner:

Dehulled and milled *Vicia faba* seeds were dispersed in tap water at an ambient temperature and extracted at pH 7.5. Acetylation occurred during the extraction step by adding of acetic anhydride and maintaining the adjusted pH by adding NaOH solution. In all cases the insoluble residues were removed by centrifugation and the extracted proteins were precipitated at their isoelectric point (pH 4.0), washed with tap water, separated from the supernatant, neutralized and dried.

A more detailed description of this procedure is given in SCHNEIDER and co-workers (1985).

1.2. Preparation of samples

The material to be tested was stirred, portion by portion, for 10 min in distilled water in a beaker, the pH was then adjusted to 7.0. Thereafter the suspension was stirred for two hours with maintaining constant the pH. Finally the suspension was pressure filtered. Border conditions (geometry of vessel and stirrer, stirring speed, temperature, time, pH, and pressure filtration) were kept constant for every test program as far as technically possible. Samples of 100 cm^3 were prepared.

1.3. Viscometry

In every case, 45 min after the end of stirring the measurements were carried out with the rotating viscosimeter Rheotest 2, a coaxial cylinder system with a gap of 1 mm (VEB MLW Prüfgerätewerk, Medingen) in the measuring system S1.

1.4. Thermal treatment

1.4.1. Exposure to different temperatures. Samples were thermally treated for 20 min at a temperature between 30 °C and 100 °C. Thirty min after the thermal exposure the apparent viscosity was determined.

1.4.2. Exposure for different times. Samples were thermally treated at 100 °C the exposure time being varied between 1 and 60 min.

1.4.3. Cyclic temperature test. Each sample was heated continuously in the rotating viscosimeter from 25 °C up to 80 °C and re-cooled. Every 5 degrees the apparent viscosity was registered at a constant rate of shear.

1.5. Dependence on time

Changes of the apparent viscosity with exposure time at room temperature were registered for 32 hours with two chosen protein charges.

1.6. Flow curves

Flow curves were registered progressively and regressively on isothermal conditions directly after the viscosity measurements.

The computer aided analysis of the flow curve was realized by a program system based on the powerlaw of Ostwald-de Waele. This was carried out on the minicomputer KRS 4200 (VEB Kombinat Robotron) using the display unit DPE 4054 (Zentralinstitut für Kernforschung, Rossendorf) for graphic representation and for realization of a dialogue mode with light pencil aid.

Values for α, which were determined by the rotating viscosimeter, were arranged according to the tests for each rate of shear and taped together with the corresponding step numbers. On the base of the step scheme and the apparatus constant which are accumulated in the computer and with reference to the used measuring system, the coordination of the shear rate D_r and the calculation of the shearing stress τ_r and of the dynamic viscosity η for the corresponding steps, are carried out. After a logarithmic transformation of the values of D_r, τ_r and η, D_r in dependence on τ_r is represented graphically point by point on the display. Thereby the possibility is given to evaluate before the utilization of the tests, the reliability and the quasi-linear behaviour of the series of measured values and to select or exclude parts of the series. Moreover, it is possible to realize a curve analysis interval by interval for flow curves which are quasi-linear only for limited parts. Borders of an interval can be defined in touching once or several times two points of the curve each with the light pencil, thereby asking step by step for a linear regression and for a curve analysis. The results of the levelling of the curve by the functions

$$\ln D_r = a_1 + b_1 \ln \tau_r$$

and

$$\ln \eta = a_2 + b_2 \ln \tau_r$$

are printed before and after the back transformation. Moreover, the graph of the starting values compared with the regression function, enables a review over the quality of the levelling of the curve and the reliability of the results.

2. Results and discussion

Figure 1 shows that the increase of the apparent viscosity with increasing protein concentration is generally not linear and is different for the individual *Vicia faba* protein isolates (VfI). These differences are particularly marked between nonacetylated *(A* and *B)* and acetylated protein isolates *(C* and *D);* in addition to that, the concentration dependent behaviour of the viscosity is influenced by the production conditions of the VfI (laboratory or small scale pilot plant). In the light of these partially considerable differences it seemed

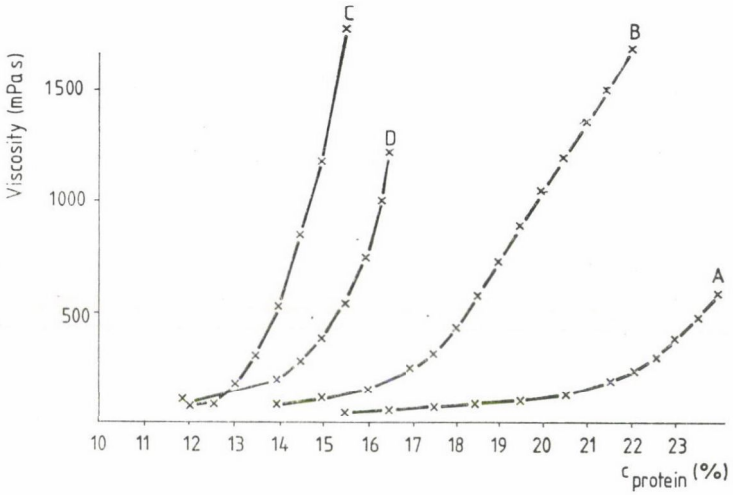

Fig. 1. Apparent viscosity η ($D_r = 145.8\ s^{-1}$) of suspensions of *Vicia faba* protein isolates (*A* and *B*) and of the corresponding acetyl derivatives (*C* and *D*). Dependence on protein concentration (*c*)

to be not advisable to use as a base for comparing studies a defined protein concentration but a comparable starting viscosity. This was the more advantageous since the order of magnitude of viscosity changes induced by heat depends critically on the starting viscosity.

In Fig. 2 the dependence of the apparent viscosity on a heat treatment of 20 minutes at different temperatures is outlined.

Starting with a viscosity of about 20 mPa×s the acetylated VfI samples *(C* and *D)* show a great increase in the viscosity above 50 °C whilst the VfI samples *(A* and *B)* do not show this increase before 70 °C is reached.

Dependence of the apparent viscosity on the heating time at a constant temperature of 100 °C is shown in Fig. 3.

Thermal treatment up to 10 min causes a greater increase of viscosity with the acetylated VfI samples *(C* and *D)* than with the non-acetylated ones

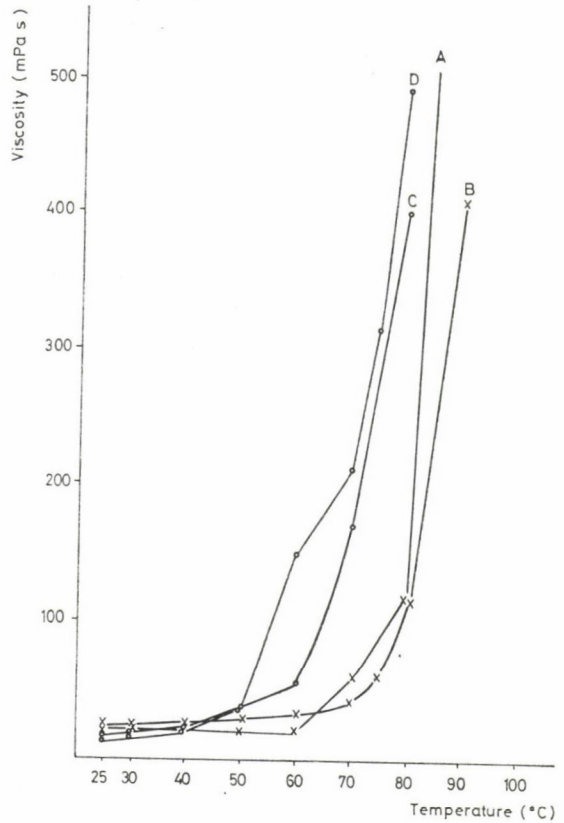

Fig. 2. Apparent viscosity η ($D_r = 437\ s^{-1}$) of suspensions of *Vicia faba* protein isolates (*A*: 16% protein and *B*: 13% protein) and of the corresponding acetyl derivatives (*C*: 10% protein and *D*: 11% protein). Dependence on a 20 minutes heat treatment at different temperatures

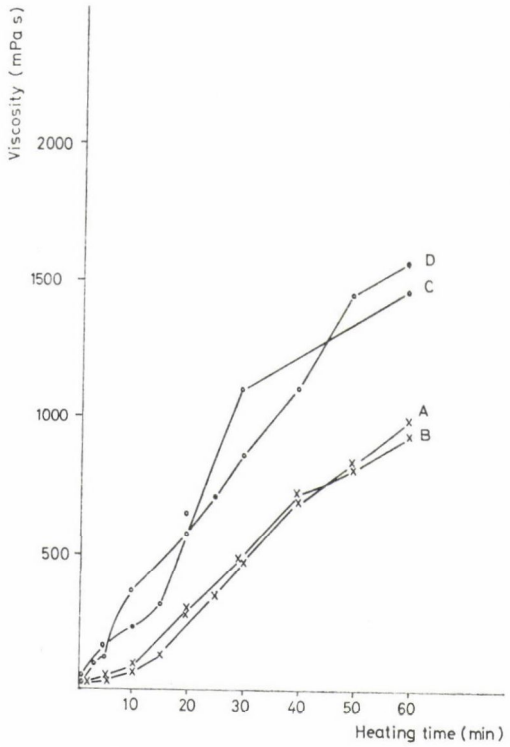

Fig. 3. Apparent viscosity η ($D_r = 145.8\ s^{-1}$) of suspensions of *Vicia faba* protein isolates (*A*: 16% protein and *B*: 11% protein) and of the corresponding acetyl derivatives (*C*: 9% protein and *D*: 9% protein). Dependence on heating time at a constant temperature of 100 °C

(A and *B)*. Increase of the apparent viscosity with increasing time of thermal treatment is almost linear. Moreover, it becomes clear that the different conditions of production of the protein isolates *A* and *B* versus *C* and *D* do not affect the viscosity behaviour which is dependent on the time of thermal treatment. The steepness of the curves increases with increasing starting viscosity as shown in Fig. 4.

When suspensions of comparable starting viscosities are subjected to a cyclic temperature test, VfI *(A)* and the acetylated VfI *(C)* behave differently. Figure 5 makes clear that the decrease of viscosity in product *C* which is caused by the increase of temperature will be superimposed by beginning gel formation above 50 °C.

A strong increase of viscosity is observed which goes on during the cooling phase. At the same conditions product *A* shows no gelling.

The order of magnitude of viscosity changes due to a cyclic temperature load depends to a high degree on the starting viscosity of the samples. A small increase of the starting viscosity causes a disproportionally higher increase in viscosity during heating and recooling. This is shown in Fig. 6 for product *C* and in Fig. 7 for product *A*, respectively.

One fact is noteworthy: when a sufficiently high starting viscosity is chosen, even the non-acetylated VfI shows gelling already during the heating period. This becomes clear from the increase of viscosity above 70 °C. Different conditions of production of the protein isolates do not affect essentially the viscosity behaviour in the cyclic temperature test. This is valid for non-acetylated as well as for acetylated VfI. Investigations in this connection have been carried out. As shown in Fig. 8 an increasing starting viscosity of product *C* causes an increasing dependence of the apparent viscosity on the holding time of the solution, too.

With comparable starting viscosities of product *A* a dependence of the apparent viscosity on the holding time could not be detected.

Figure 9 shows the measured values of a flow curve as visible on the display and their partition in two partial series; in Fig. 10 we have plotted the starting values of a partial series compared with the slope of the regression function.

All flow curves elaborated in the course of the investigations here reported were separated each into a partial series for a low and a high shear rate, both partial series were calculated separately. The calculated values for the slope of the curve were marked by tg α_1 for the part of the flow curve with a low shear rate and by tg α_2 for the part of the flow curve with a high shear rate, respectively. If flow curves in the double-logarithmic coordinate system (lg D_r versus lg τ) are linear, then

$$\text{tg } \alpha_1 = \text{tg } \alpha_2$$

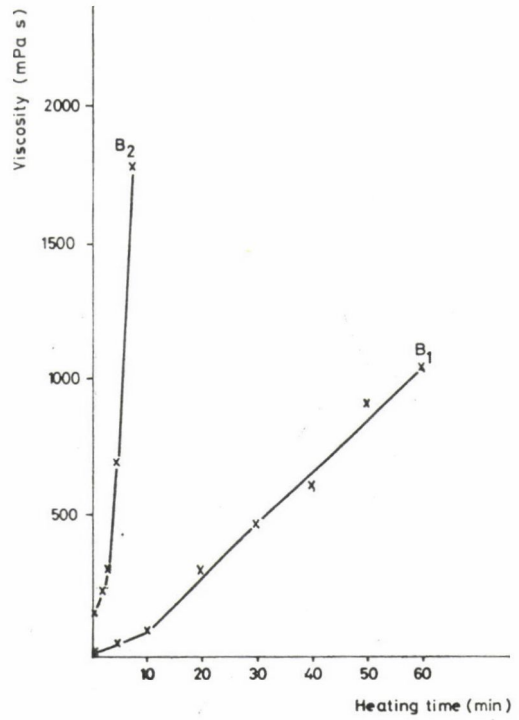

Fig. 4. Apparent viscosity η ($D_r = 145.8\ s^{-1}$) of suspensions of *Vicia faba* protein isolates (B_1: 11%; B_2: 16%). Dependence on the heating time at a constant temperature of 100 °C with regard to the starting viscosity

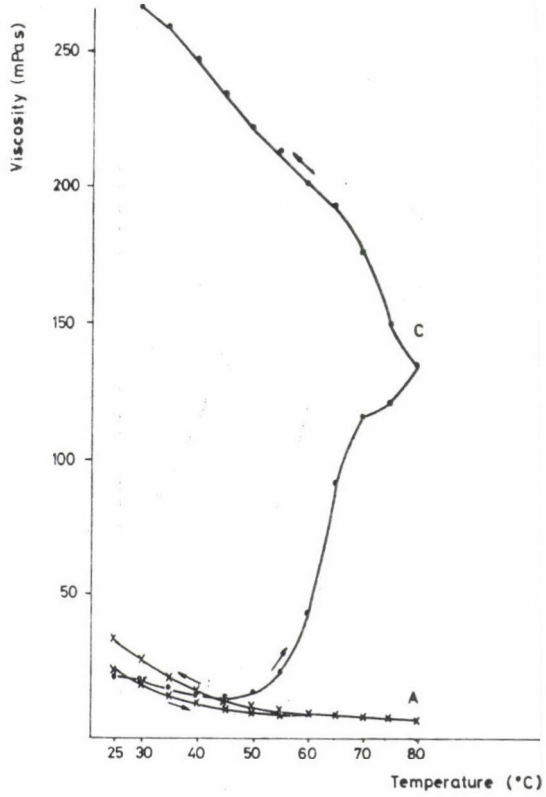

Fig. 5, Apparent viscosity η ($D_r = 145.8\ s^{-1}$) of suspensions of *Vicia faba* protein isolate (*A*: 17% protein x——x) and of the corresponding cyclic derivative (*C*: 11% protein o—— o) as a function of a cyclic temperature test

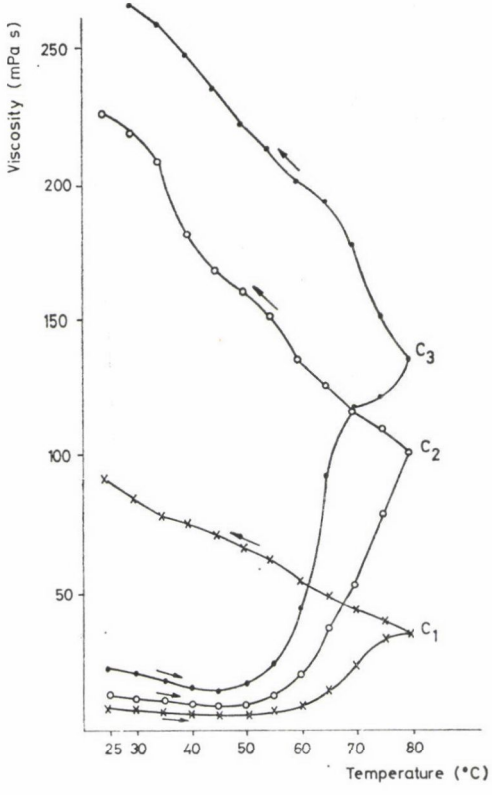

Fig. 6. Apparent viscosity η ($D_r = 656\ s^{-1}$) of suspensions of acetylated *Vicia faba* protein isolates (C_1: 9% protein; C_2: 10% protein; C_3: 11% protein) as a function of a cyclic temperature test with regard to the starting viscosity

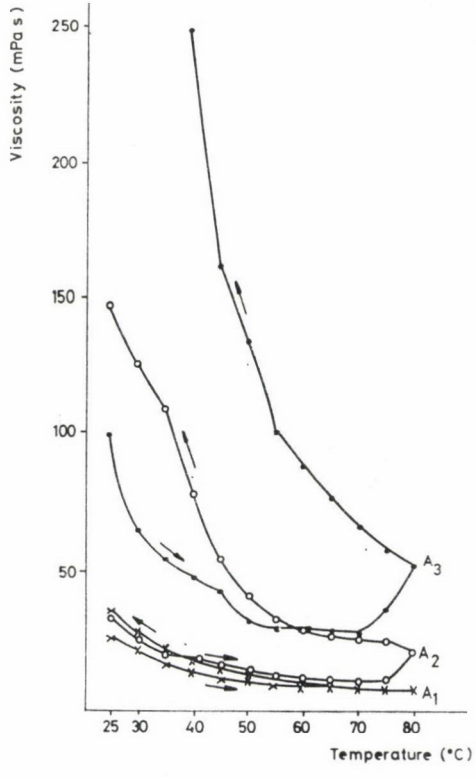

Fig. 7. Apparent viscosity η ($D_r = 656\ s^{-1}$) of suspensions of *Vicia faba* protein isolates (A_1: 17% protein; A_2: 18% protein; A_3: 21% protein) as a function of a cyclic temperature test with regard to the starting viscosity

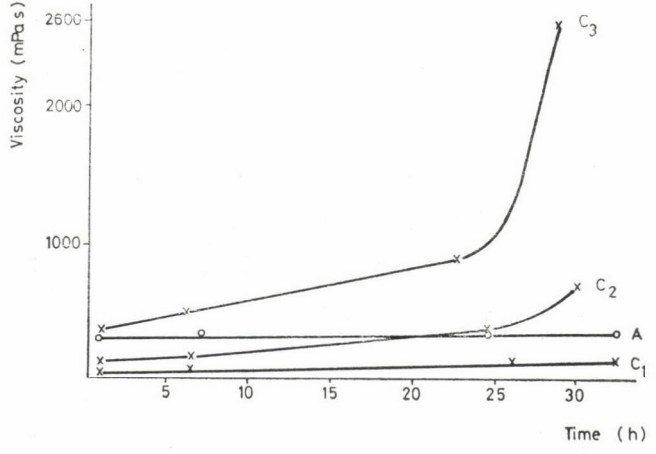

Fig. 8. Apparent viscosity η ($D_r = 145.8\ s^{-1}$) of suspensions of *Vicia faba* protein isolate (*A*: 23% protein) and of the corresponding acetyl derivatives (C_1: 12% protein; C_2: 13% protein; C_3: 14% protein). Dependence on the standing time of the solution with regard to the starting viscosity

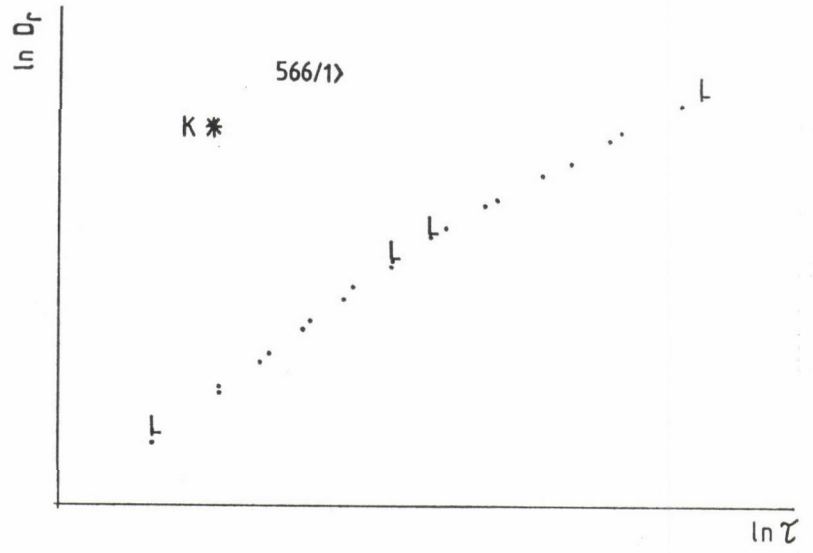

Fig. 9. Graph of the measured point of a flow curve as seen on the display, the selection of partial series included (L . . . L)

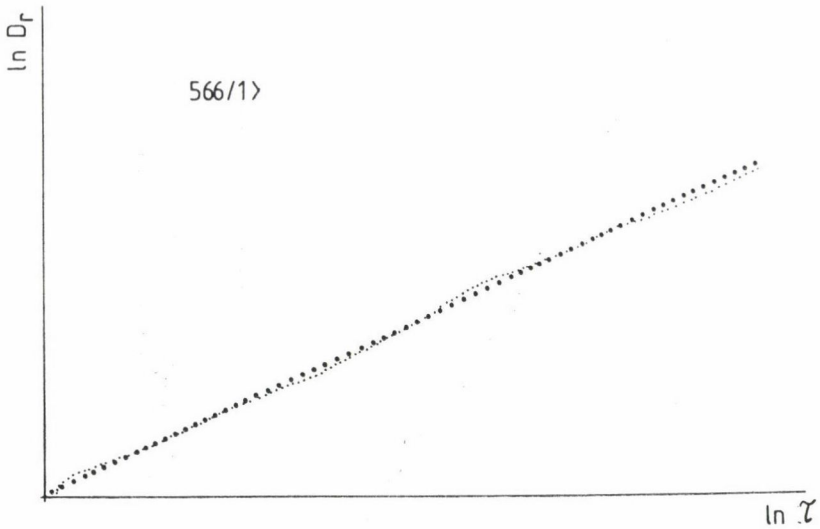

Fig. 10. Graph of the experimental values of a partial series compared with the regression function on the display. ·····: experimental values; • • • •: regression function

is valid. This is characteristic for a Newtonian flow ($\mathrm{tg}\,\alpha = 1$) and a pseudoplastic flow ($\mathrm{tg}\,\alpha > 1$), respectively. For nonlinear flow curves the following correlations are valid:

$\mathrm{tg}\,\alpha_1 > 1;\ \mathrm{tg}\,\alpha_2 = 1$ — Bingham flow behaviour,
$\mathrm{tg}\,\alpha_1 > 1;\ \mathrm{tg}\,\alpha_2 > 1;\ \mathrm{tg}\,\alpha_1 > \mathrm{tg}\,\alpha_2$ — plastic flow behaviour.

In Fig. 11 the $\mathrm{tg}\,\alpha$ is represented as a function of protein concentration.

For pseudoplastic systems the curves for $\mathrm{tg}\,\alpha_1$ and $\mathrm{tg}\,\alpha_2$ are superposed. Particularly for the acetylated product *D* it becomes clear that with increasing protein concentration the pseudoplastic part increases first. Above a protein concentration of 15 per cent the material under investigation exhibits increasingly plastic flow properties. This can be seen by the increasing difference between $\mathrm{tg}\,\alpha_1$ and $\mathrm{tg}\,\alpha_2$ with further increase of the $\mathrm{tg}\,\alpha$-values at the same time. Moreover, it becomes clear that in a comparable concentration range the change of the flow characteristics of the non-acetylated product is less drastic.

In Fig. 12 and Fig. 13 the dependence of $\mathrm{tg}\,\alpha$ on temperature and on heating time, respectively, are represented.

It can be estimated that using the described method for the evaluation of flow curves, it is possible to recognize changes in the basic flow properties induced by concentration and temperature influence and to follow up their trend.

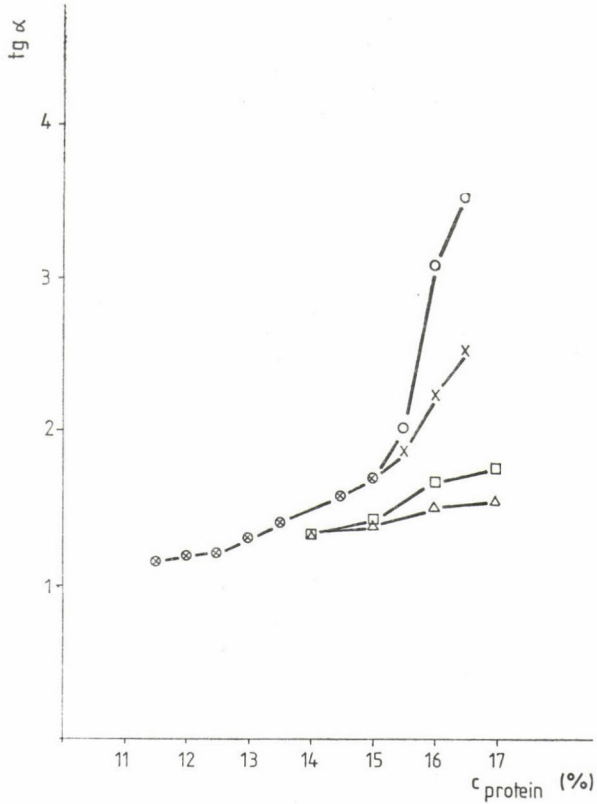

Fig. 11. The increases of tg α of the flow curves of suspensions of *Vicia faba* protein isolate, product *B*, and of the corresponding acetyl derivative, product *D*, in dependence on protein concentration *c*. Product *B*: △—△ low rate of shear; □—□ highrate of shear. Product *D*: x—x low rate of shear; ○—○ highrate of shear

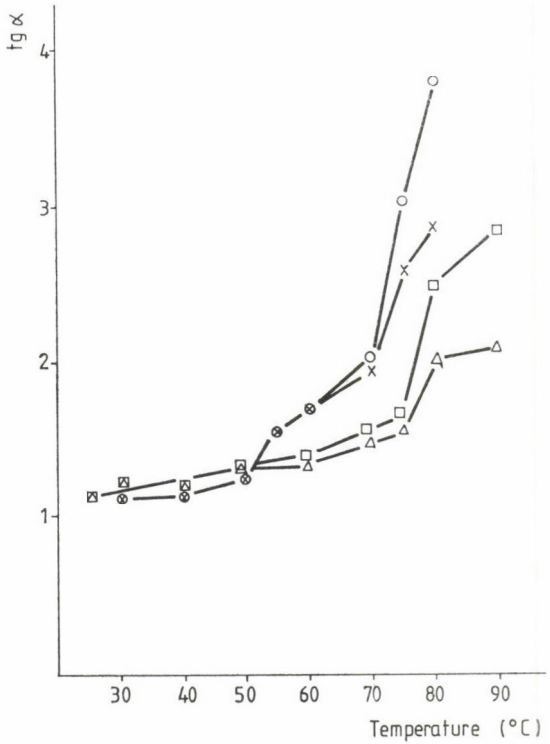

Fig. 12. Increase of tg α of the flow curves of suspensions of *Vicia faba* protein isolate, product *B*, and of the corresponding acetyl derivative, product *D*, in dependence on heating temperature. For legends see Fig. 11

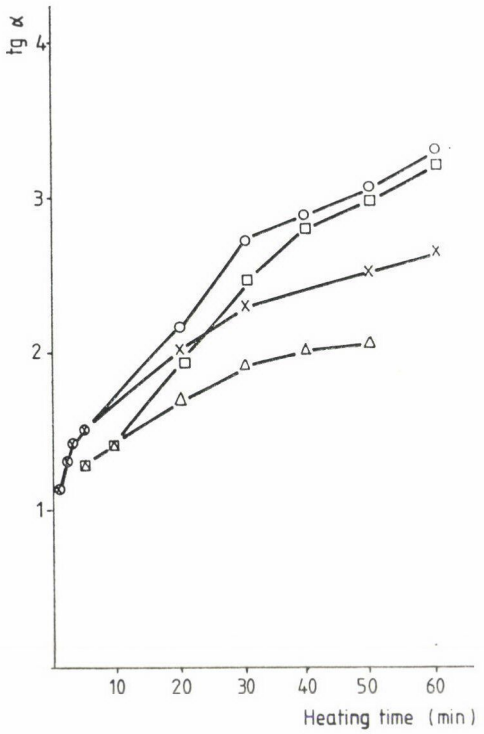

Fig. 13. Increase of tg α of the flow curves of suspensions of *Vicia faba* protein isolate, product *B*, and of the corresponding acetyl derivative, product *D*, in dependence on heating time. For legends see Fig. 11

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DETERMINATION OF PHYLLOQUINONE (VITAMIN K_1) IN RAW AND PROCESSED VEGETABLES USING REVERSED PHASE HPLC WITH ELECTROFLUOROMETRIC DETECTION

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The phylloquinone (PK) content of a number of vegetables was determined by reversed phase HPLC combined with fluorometric detection after post-column electrochemical reduction. The lipids were extracted with n-hexane from the material, which was homogenized by means of an Ultra-Thurrax mixer.

Neither cooking nor γ-irradiation of the vegetables lowered the PK content, nor did the PK content of commercially available vegetable preparations, dried or deep-frozen, in cans or glass containers, differ significantly from the content of fresh vegetables.

Keywords: phylloquinone, reversed phase HPLC, electrofluorometric detection, vegetables

The uptake of phylloquinone (vitamin $K_{1(20)}$, PK; Fig. 1) from food is probably the most important source for vitamin K in man. Although the daily requirement appears to be low (0.5–1.0 μg per kg body weight) (FRICK et al., 1967) and deficiencies are rarely encountered with normal diets, it is still important to get an insight into the daily uptake, especially for patients receiving anticoagulants of the coumarin type. Since coumarins are the antagonists of vitamin K in the blood-clotting process, the uptake of relatively high amounts of PK from food may potentially interfere with the anticoagulant therapy resulting in a higher risk of thrombosis for the patient. As a part of the study of the influence of the uptake of vitamin K from food on the stability of oral anticoagulant therapy, the vitamin K content of a number of common Dutch vegetables was determined.

Recently a few methods for the analysis of PK in food using off-line multidimensional high-performance liquid chromatography (HPLC) with UV detection have been published (THOMPSON et al., 1978; SHEARER et al., 1980). Although these methods seem to give satisfactory results, there are some major drawbacks. One is the necessity for the full-time use of two complete chromatographic systems, which is rather expensive. Furthermore, the fractions

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to be collected from the semi-preparative normal phase system have to be as small as possible in order to eliminate interferences in the final reversed phase system. This implies that the analysis generally has to be limited to one vitamin K at a time. A relatively large number of manipulations with the samples are needed, which may give rise to experimental errors, if no suitable internal standard is used. Furthermore, the method is too laborious and time-consuming for the analysis of large numbers of samples.

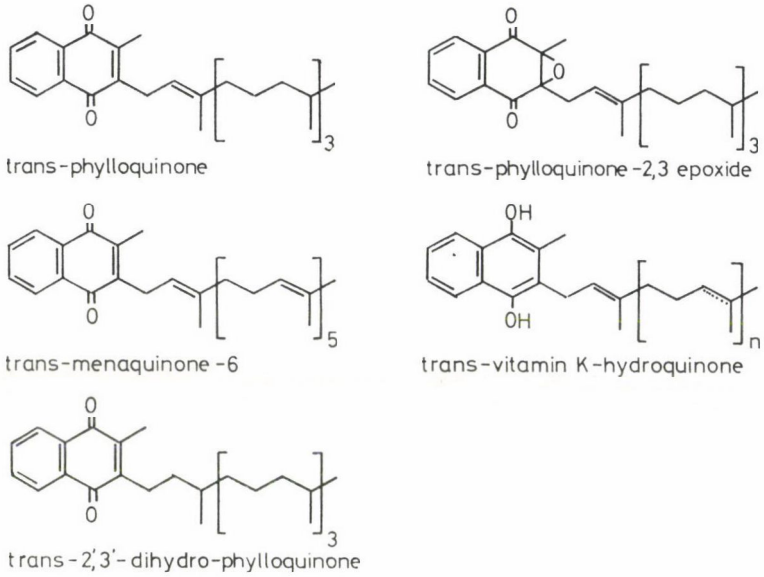

Fig. 1. Structural formulae of the vitamins K of interest

Therefore we applied the method as developed for the determination of vitamins K in human plasma (LANGENBERG & TJADEN, 1984a, b) to this study of vitamin K in vegetables directly. The method is based on the fluorometric detection of the corresponding hydroquinones, which are formed post-column by quantitative electrochemical reduction of the vitamins K. Owing to the high fluorescence of the hydroquinones a highly selective and sensitive detection is obtained.

Since our main interest was the vitamin K content of normal diets, our study was not limited to raw, fresh vegetables but we also investigated the effect of cooking, deep-freezing, drying, heat sterilization and γ-irradiation. γ-Irradiation is a powerful technique to extend the keeping quality of fresh vegetables. Its use in the preservation of food is permitted for a number of products. Data in literature concerning the stability of vitamin K towards ir-

radiation are controversial (RICHARDSON et al., 1956; METTA et al., 1959; RICHARDSON, 1960; REBER & MALHOTRA, 1961; MATSCHINER & DOISY, 1966) probably because of the lack of selective methods of analysis. Furthermore, the irradiation doses used in these studies were fairly high, mostly 27.9 or 58 kGy. Nowadays 2 kGy is considered to be the most suitable dose, since the radurization is effective while the organoleptic properties of the vegetables are not affected. In this study also an irradiation dose of 10 kGy was applied, because the Joint Expert Committee of FAO and WHO has accepted food irradiation up to this dose.

1. Materials and methods

1.1. Apparatus

The liquid chromatograph was constructed from commercially available and custom-made parts and consisted of a thermostatted glass-eluent reservoir (25 °C), a dual piston constant-flow pump (Model 2150, LKB Products, Bromma, Sweden), an injection device with a 20 μl loop (Model 7125, Rheodyne, Berkeley, CA, USA) and a thermostatted column (stainless steel precision-bore tubing, 100×3.0 mm i.d.). The detection system consisted of a dual electrochemical detector cell (Model 5010 Coulochem, ESA, Bedford, MA, USA) coupled to a polarograph (Model E 445, Bruker, Brussels, Belgium) used as a reactor and a fluorometer of the double monochromator type (Model RF 530, Shimadzu, Kyoto, Japan) operating at an excitation wavelength of 320 nm and an emission wavelength of 430 nm. Chromatograms were registered on a flat-bed recorder (Model 80-8, Kipp & Zn, Delft, The Netherlands). Peak areas were calculated with a computing integrator (Chromatopac C-ElB, Shimadzu, Kyoto, Japan).

1.2. Materials

Phylloquinone (PK), menaquinone-4 (MK-4) and menaquinone-6 (MK-6) and phylloquinone-2,3-epoxide (PK-ep) were kindly donated by Hoffmann-La Roche (Mijdrecht, The Netherlands). 2',3'-Dihydrovitamin $K_1(_{20})$ (K_1-/I-H_2) was synthesized from PK as described elsewhere (LANGENBERG & TJADEN, 1984b). All organic solvents were of analytical grade (J. T. Baker, Phillipsburg, NJ, USA). Water was purified by means of a Milli-Q-Water Purification System (Millipore, Bedford, MA, USA). Hypersil-MOS (5 μm) was purchased from Shandon (London, UK). Columns were slurry-packed by means of an air (pressure) driven booster pump (Model DSHF 302, Haskel, Burbank, CA, USA) according to a method described elsewhere (TJADEN et al., 1980).

1.3. Chromatographic conditions

A mixture of 92.5% methanol and 7.5% water containing 0.03 mol of sodium perchlorate as supporting electrolyte was used as the mobile phase. The removal of oxygen from the mobile phase, which is essential for electrofluorometric detection, was accomplished by continuously bubbling nitrogen, made oxygen-free with a solution of pyrogallol in potassium hydroxide and presaturated with the mobile phase.

1.4. Preparation of the samples

1.4.1. Raw vegetables. The vegetables were prepared as for consumption, meaning that only the edible parts were analysed. The material was chopped up with a sharp stainless-steel knife and the parts were mixed thoroughly. Three aliquots of about the same weight were analysed. The samples were weighed into snap-cap bottles of about 65 cm^3. Under subdued light 10 cm^3 of isopropanol was added as well as the internal standard MK-6 (100 μl of a solution of 100 $\mu g\ cm^{-3}$ in methanol). The sample was homogenized completely with an Ultra-Thurrax mixer. Mixing was repeated two times after the addition of two aliquots of 10 cm^3 of n-hexane. Phase separation was initiated by the addition of 10 cm^3 of water after which the bottle was centrifuged 10 min×1000 g. An aliquot of 5 cm^3 of the upper layer was removed and evaporated to dryness at ambient temperature using a rotary evaporator. The residues were redissolved completely in 5 cm^3 of methanol of which aliquots of 20 μl were injected onto the HPLC column. During the extraction the samples were shielded from light as much as possible.

1.4.2. Cooked vegetables. The method is basically the same as for raw vegetables, only in this case the three aliquots of each sample were cooked in the snap-cap bottles in an appropriate amount of water. After cooling the water was removed and analysed. The change in weight of the vegetables was registered. The samples were homogenized and extracted as described above.

1.4.3. γ-Irradiation. The vegetables were cleaned and chopped up. Three portions were taken and were packed in polyethylene bags. Each of the bags was placed in a different large beaker. One of these beakers was placed outside the irradiation chamber, the other two were placed on turntables close to the ^{60}Co-source. The capacity of the source was 114.7 PBq, while the dose-rate amounted to 2.64 kGy h^{-1}. The absorbed doses were calculated from the dosimeter value (perspex) and were determined to be 2.1 and 10.5 kGy, respectively. The samples were assayed as described for raw vegetables. Standard solutions of PK in methanol with concentrations of 10, 100 and 1000 ng cm^{-3} and mixtures of some vitamins K in *n*-hexane, methanol and water in polyethylene tubes were also exposed to γ-irradiation, next to the vegetables. For analysis the hexane and methanol samples were evaporated and the residue

was dissolved in 200 μl of methanol, whereas the aqueous solutions were extracted with *i*-propanol and *n*-hexane.

1.4.4. Commercially available products. A number of deep-frozen, glass-packed, canned and dried vegetables were assayed. These products were prepared as is usual for consumption and were analyzed as described for cooked vegetables.

2. Results

2.1. Chromatography

The reversed phase system with selective detection could be applied to the assay of the raw extracts of the vegetables. Owing to the relatively high concentration of PK in the samples and the low total amount of lipids extracted from the material, the dried residues of the sample extracts could be dissolved

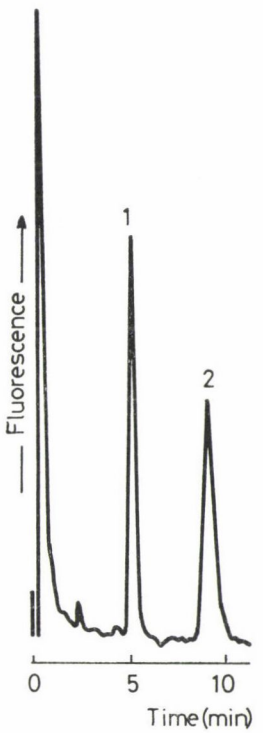

Fig. 2. Chromatogram of an extract of raw kale. Conditions: stationary phase, Hypersil/MOS (5 μm); mobile phase, MeOH/H_2O 92.5/7.5 with 0.03 mol $NaClO_4$; flow-rate: 0.8 cm^3min^{-1}; potential applied to the coulometric cell: —500 mV; excitation wavelength: 320 nm; emission wavelength: 430 nm. 1: PK (13.9 ng) and 2: MK-6 (9.3 ng)

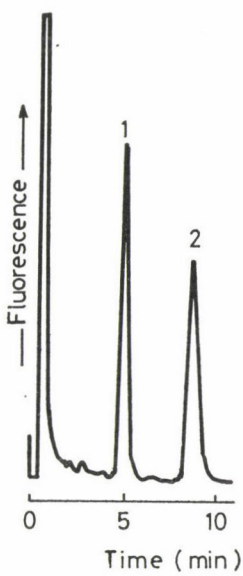

Fig. 3. Chromatogram of an extract of cooked spinach. Conditions as for Fig. 2. 1: PK (8.7 ng) and 2: MK-6 (10.4 ng)

completely in 5 cm^3 of methanol. The detection limit for PK in this system amounting to 50 pg is far below the amounts of PK which were injected onto the column. As can be seen from Figs. 2, 3, 4 and 5 only a few peaks are observed in the chromatograms indicating the high selectivity of the applied detection method. The system operated for months without significant loss of column efficiency enabling us to assay samples with a throughput of 5 samples per hour. The daily sample throughput is limited by the necessity of regenerating the electrodes for about 8 hours per day in the oxidative mode. Continuously operating the electrodes in the reductive mode will lead to a change in the relationship between the fluorescence yield and the applied potential causing gradual loss of sensitivity. When using two coulometric cells connected to the column through a switching-valve, the system can be operated full-time, using an automatic sample injector.

In our study the resolution between PK and the other components detectable with this system is higher than strictly necessary. If desired, the analysis time can be shortened considerably by increasing the flow-rate or the methanol content of the mobile phase, or by using K_1-(I-H_2) as an internal standard, which is eluted directly after PK. Unfortunately, we observed a small peak with about the same retention time as K_1-(I-H_2) in a few vegetable

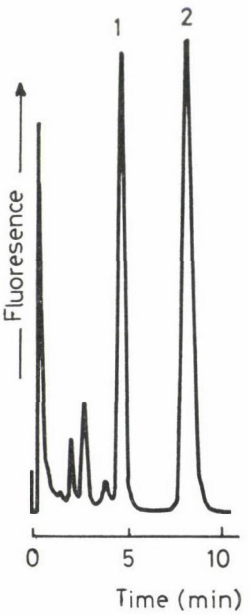

Fig. 4. Chromatogram of an extract of radurized raw red cabbage (2.1 kGy). Conditions as for Fig. 2. 1: PK (9.8 ng) and 2: MK-6 (20.2 ng)

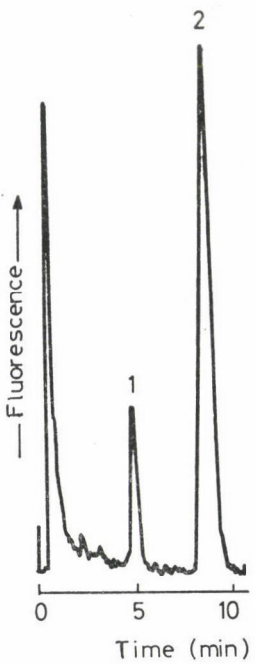

Fig. 5. Chromatogram of an extract of raw leek. Conditions as for Fig. 2. 1: PK (1.6 ng) and 2: MK-6 (11.0 ng)

extracts, whereas no peaks with a retention time corresponding to that of MK-6 were observed.

We also investigated the possibility of using normal phase HPLC with UV detection for the assay of the samples. This appeared to be applicable only to a limited number of vegetables, since many interferences with long retention times were noted in the chromatograms resulting in long analysis times. Furthermore, the efficiency of the silica column decreased quite rapidly due to the strong adsorption of polar components present in the extracts.

2.2. Extraction procedure

A few methods of extraction were compared using a large homogeneous sample of raw cauliflower. It appeared that Soxhlet extraction with acetone was very effective but rather time-consuming when applied to large numbers of samples. We used this method as the reference for the evaluation of other extraction procedures. Simple extraction procedures, such as ultrasonification of the sample with acetone, chloroform–methanol or *n*-hexane–*i*-propanol lead to low and irreproducible recoveries. Homogenization using a Potter-tube homogenizer or an Ultra-Thurrax mixer proved to give equally high and also reproducible recoveries. We prefer the use of an Ultra-Thurrax mixer, since it is capable of grinding even the toughest materials, such as raw white cabbage. This is far more difficult with the Potter equipment.

The internal standard is merely used for the correction of small differences in phase ratios and losses due to the manipulations, since it is impossible to incorporate the standard into the materials in the same way as PK. The extraction of PK from the cells of the vegetables with the Ultra Thurrax mixer proved to be nearly quantitative. Repeated extraction of the residue, either with the same method or by Soxhlet extraction, did not show a significantly different PK to MK-6 ratio.

Although the high sensitivity of the method allows the use of very small sample amounts, we prefer to extract larger quantities (5–10 g). Owing to the inhomogeneous distribution of PK in the plant, the standard deviation of 3 assays of 1 g of raw cauliflower was as high as 8% whereas with samples of 10 g the relative standard deviation was about 3%.

2.3. Quantification

The PK content of the different vegetables was calculated from the PK to MK-6 peak area ratio. The method proved to be linear from at least 100 pg to 1000 ng directly injected PK. The linearity of the method for the assay of extracts was confirmed by standard addition of PK to raw cauliflower samples. The coefficient of correlation of the PK to MK-6 peak area ratio was determined to be 0.99998.

The precision of the method was determined by extracting 5 aliquots of a mass of raw cauliflower which was likely to be homogeneous after grinding with a kitchen blendor. The recovery of the extraction appeared to be about 92.5%. The coefficient of variation for the determination of PK in raw cauliflower was 3.2% (n = 5) and for softer tissues such as spinach even as low as 2.3% (n = 5).

Table 1

The phylloquinone content of a number of vegetables (μg per 100 g), *before and after various manipulations for preparation or preservation*

Vegetable	Raw	Cooked	Deep-frozen	Canned or potted	Irradiated	
					2.1 kGy	10.5 kGy
Red beet	0	0	—	—	—	—
Potato	1	1	—	—	1	1
Turnip cabbage	2	2	—	—	—	—
Tomato	6	—	—	—	5	6
Carrot	11	11	—	10	11	10
Leek	18	17	—	—	20	14
Cucumber	22	—	—	—	19	20
Sauerkraut	25	22	—	—	24	26
Cauliflower	25	23	—	—	25	24
Peas	33	33	34	35	—	—
Beans	53	51	43	45	48	44
White cabbage	55	46	—	—	55	59
Red cabbage	57	47	—	—	56	53
Lettuce	123	—	—	—	—	—
Pointed cabbage	175	143	—	—	183	166
Sprouts	175	145	165	—	208	203
Sprouts (outer leaves)	475	395	415	—	—	—
Turnip tops	192	—	—	—	—	—
Broccoli	205	150	270	—	213	215
Green cabbage	216	180	—	—	224	228
Endive	231	159	—	—	270	265
Purslane	381	545	—	—	—	—
Spinach	385	562	561	609	393	388
Kale	817	464	475	505	780	765

Contents listed are mean values (n = 5), except for the irradiation experiments (n = 1). Student's *t*-test was used to establish significant differences with $P < 0.02$ as the minimum level of significance

In Table 1 the phylloquinone contents of a number of vegetables, commonly consumed in The Netherlands, are summarized. It should be realized that the PK content of a vegetable is influenced by many factors such as genetic and environmental factors. The cauliflower is an illustrative example. THOMPSON and co-workers (1978) found less than 5 μg 100 g^{-1}, SHEARER and co-workers (1980) found 27 and we found 25 μg 100 g^{-1}. Generally the values shown in Table 1 are in a good agreement with those found by Thompson and Shearer. Only the values listed by OLSON (1980) differ to some extent, but of course the method of analysis was quite different.

From the results presented in Table 1 it is clear that cooking of vegetables does not significantly alter the PK content. As expected from the poor solubility of PK in water, the amounts found in the cooking water (Fig. 6) were in the nanogram range corresponding to a loss of far less than 0.1%. Changes in the PK content during cooking can be attributed to the change in weight, due to the uptake of water (e.g. kale) or the loss of water (e.g. spinach and purslane).

Due to the relatively high thermostability of PK no degradation during cooking was expected. The stability towards γ-irradiation was much more difficult to predict. Irradiation of pure solution of vitamins K leads to degradation as is shown in Figs. 7a, b and c. The results suggest that the vitamins K are more stable when dissolved in *n*-hexane than in more polar solvents such as methanol or water and that the more polar vitamins K are degraded faster than the less polar vitamins K. A more detailed study is required to further substantiate such suppositions, but this was beyond the scope of the present study. With these observations in mind it is interesting to note that we did not observe a significant decrease in the PK content of the vegetables as a result

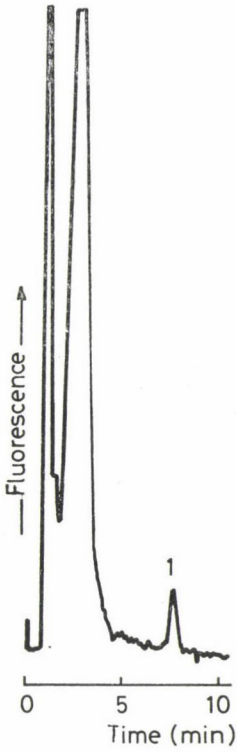

Fig. 6. Chromatogram of the extract of water in which spinach was cooked (see Fig. 3.). Conditions as for Fig. 2. 1: PK (0.56 ng)

of γ-irradiation, not even with the high dose of 10.5 kGy. With this unrealistic high dose for fresh vegetables some of these commodities showed loss of organoleptic properties and were sloppy. Apparently PK is stabilized in the vegetable, a phenomenon that is often observed in irradiation experiments (TOBBACK, 1979). As far as the vitamin K content is concerned, there are no objections to radurization of vegetables by means of γ-irradiation.

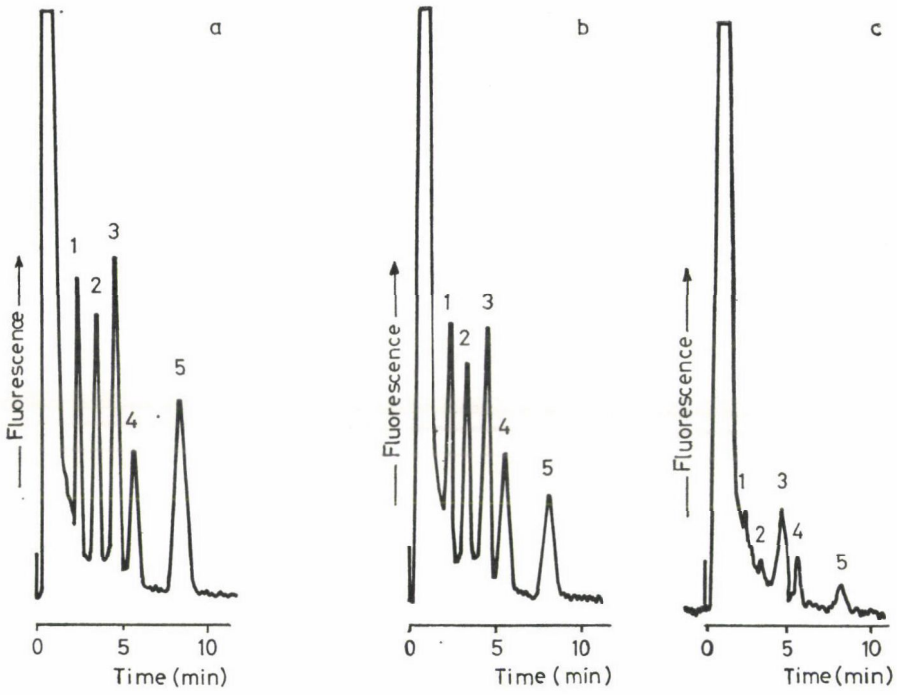

Fig. 7. The effects of γ-irradiation on a mixture of several vitamins K in methanol. Conditions as for Fig. 2, except for potential applied to the coulometric cell: —1750 mV. a: not irradiated, 1: MK-4 (6.1 ng) 2: PK-ep (3.0 ng), 3: PK (3.7 ng), 4: K_1-(I-H_2) (1.5 ng) and 5: MK-6 (4.6 ng); b: irradiation dose 2.1 kGy and c: irradiation dose 10.5 kGy

After correction for the change in weight due to the loss or uptake of water the PK content of deep-frozen vegetables was about the same as in fresh material. Only for sprouts we sometimes found higher values for the deep-frozen product. This may be explained by the fact that the deep-frozen sprouts mostly still possess their outer leaves, which contain the highest PK amount. With fresh sprouts these are mostly removed prior to consumption, because they are too tough.

Heat sterilization is used for vegetables in cans and glass containers and this apparently does not cause degradation of PK, which could be predicted from its thermostability.

Freeze-drying of food has the disadvantage that it leads to loss of vitamins (MATSCHINER et al., 1967). Nowadays vegetables are dried by a different process for which it is claimed that there is no influence on the vitamin content. The results of the present study confirm this for PK.

Finally we observed that PK was not degraded when using a microwave oven for rapid warming up of the food.

Since *cis-* and *trans*-isomers cannot be separated in reversed phase systems, we investigated the possible presence of cis-PK by combining a normal phase HPLC system with electrofluorometric detection (LANGENBERG & TJADEN, 1984a). We did not detect any cis-PK which means that the total PK values found with our reversed phase system are truly trans-PK concentrations. We did find low amounts of PK-ep in some of the vegetables, although it was never more than 1 or 2% of the PK content and therefore no essential contribution to the vitamin K status is to be expected from these amounts of PK-ep.

We could not contribute any other peak to known vitamins K which is in agreement with the general opinion that only trans-PK occurs in plants.

3. Conclusions

The application of reversed phase HPLC with electrofluorometric detection simplifies the assay of PK in vegetables considerably, since no clean-up or fractionation of the raw extracts is necessary. Owing to the high sensitivity and selectivity of the detection method very small samples can be used for the assay but as a result of the inhomogeneous distribution of PK in vegetables larger sample amounts lead to more reproducible results.

Cooking or γ-irradiation up to 10.5 kGy does not lower the PK content as compared with fresh vegetables. Irradiation of pure solutions of vitamins K shows rapid degradation. With respect to the PK content commercially available vegetable products in cans or glass containers, dried or deep-frozen are not inferior to fresh vegetables.

Normal diets will supply sufficient PK to man to meet the daily requirements, provided that the bioavailability of PK from vegetables is adequately high.

*

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POLYPHENOL OXIDASE (PPO) AND PEROXIDASE (POD) ENZYME ACTIVITIES AND THEIR ISOENZYME PATTERNS IN RIPENING FRUITS

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PPO and POD activities and their isoenzyme patterns were studied in the peel and pulp tissues of apple, avocado, sapota and mango.

During ripening, the PPO activity generally increased in all the peel tissues, whereas in pulp, it decreased in apple, increased in avocado and developed in sapota only at the ripe stage. At no stage of ripening, mango pulp exhibited PPO activity.

POD showed a peak in the activity around the climacteric stage of ripening in all the peel and pulp tissues studied.

PPO isoenzymes on the polyacrylamide gels showed an anodic trend in the movement in the case of apple peel, apple pulp and avocado peel during ripening. Unripe sapota extract exhibited no PPO activity, but revealed clear PPO isoenzyme bands when separated by disc gel electrophoresis suggesting the presence of endogenous inhibitors rather than the absence of PPO enzymes, whereas mango pulp neither exhibited PPO activity nor revealed any isoenzyme bands.

In the case of POD, intensification of the already existing bands and development of new isoenzyme bands were noticed in all the tissues during ripening.

Keywords: PPO, POD, isoenzyme, ripening fruits

The enzymes catalyzing the oxidation of polyphenols viz. PPO and POD are abundantly present in fruits and vegetables. The PPO-s are copper containing proteins and are different from peroxidases which are heme proteins. It has been well established that PPO-s are mainly responsible for the darkening or browning occurring in plant systems subsequent to bruising and during processing of fruit tissues (MAYER & HAREL, 1979). Peroxidases have been shown to contribute to the deteriorative changes in flavour, texture, colour and nutritional value in processed fruits and vegetables (HAARD, 1977).

For a better understanding of the significance of PPO and POD in fruit ripening, monitoring the enzyme activities and isoenzyme patterns in ripening fruits might be helpful. Though the PPO and POD activities and the existence of their multiple forms are documented in some fruits (MAYER & HAREL, 1979; HAARD, 1977) there are no comparative data on PPO and POD activities and their isoenzyme forms in both peel and pulp of fruits during ripening.

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1. Materials and methods

1.1. Fruits

Apples (*Malus sylvestris* — Royal Delicious Variety) were obtained from Himachal Pradesh by air transport. Avocado (*Persea americana Mill* — Fuorte variety) and mango (*Mangifera indica* — Badami variety) were obtained from the local gardens. After harvest the fruits were kept at room temperature for ripening which was 25–27 °C for apple and avocado and 28–30 °C when experiments with mango were in progress. Previous work over the last few years in our laboratory has shown that the climacteric for the fruits as judged by respiratory measurements is attained around the following periods, apple: 8, avocado: 3, sapota: 4–6 and mango: 8–10 days.

1.2. Preparation of enzyme extracts

The fruit tissues were blended in cold 0.001 mol dm^{-3} potassium phosphate buffer (pH 7.4 containing 1% w/v polyvinylpyrrolidone and used for acetone powder preparation). From four individual fruits, peel and pulp acetone dried powders, respectively, were prepared with acetone chilled to 0 °C. Enzymes from acetone powders of peel and pulp were extracted in the cold at 4 °C for 20 minutes with 10 volumes of 0.008 mol dm^{-3} potassium phosphate buffer (pH 7.4) containing 1% w/v polyvinyl polypyrrolidone. The extract was filtered through cheese cloth and centrifuged for 10 minutes at 10 000 g. The clear supernatant was used after overnight dialysis as a source of PPO and POD enzymes. For the separation of isoenzymes, the enzyme extracts were concentrated to less than half their volumes by using aquacide.

1.3. Enzyme assay

PPO and POD activities were assayed spectrophotometrically at 420 and 485 nm, respectively, as described by Wong and co-workers (1971) using catechol as substrate and Vance and Sherwood (1966). The absorbancies for both PPO and POD were read at 15 second intervals. Specific activity is expressed as units per min per mg protein (1 unit = OD change of 0.001). Each value represents the average of results obtained for four individual fruits. Protein content was estimated by Lowry's method (Lowry et al., 1951).

1.4. Eelectrophoretic separation of enzymes

Disc electrophoresis was carried out in 7.5% polyacrylamide gels (0.7 × × 8.2cm) using the formulations described by Davis (1964). Electrophoresis was carried out in TRIS-glycine buffer, pH 8.3 with a constant current of 3 mA per

tube. The PPO bands were detected with catechol and *p*-phenylene diamine staining (MONTGOMERY & SGARBIERI, 1975) followed by dipping the gels in 7% acetic acid which developed purple coloured bands. POD isoenzymes were developed with hydrogen peroxide and *o*-dianisidine (SHANNON et al., 1966) which gave orange coloured bands.

In the case of apples, while for enzyme assays fruits of optimum harvest maturity were used, for electrophoretic separations, fruits of early (E) optimum (O) and late (L) harvest maturity stages were employed.

2. Results

2.1. PPO activity in fruits during ripening

Activities of PPO in the peel and pulp tissues of apples, avocado, sapota and mango at different stages of ripening are presented in Fig. 1. (a, b, c and d). In the case of apple peel, PPO activity showed about threefold increase during ripening whereas in the pulp tissues, there was a loss of about 35% in the total PPO activity. Avocado peel and pulp exhibited about twofold increase

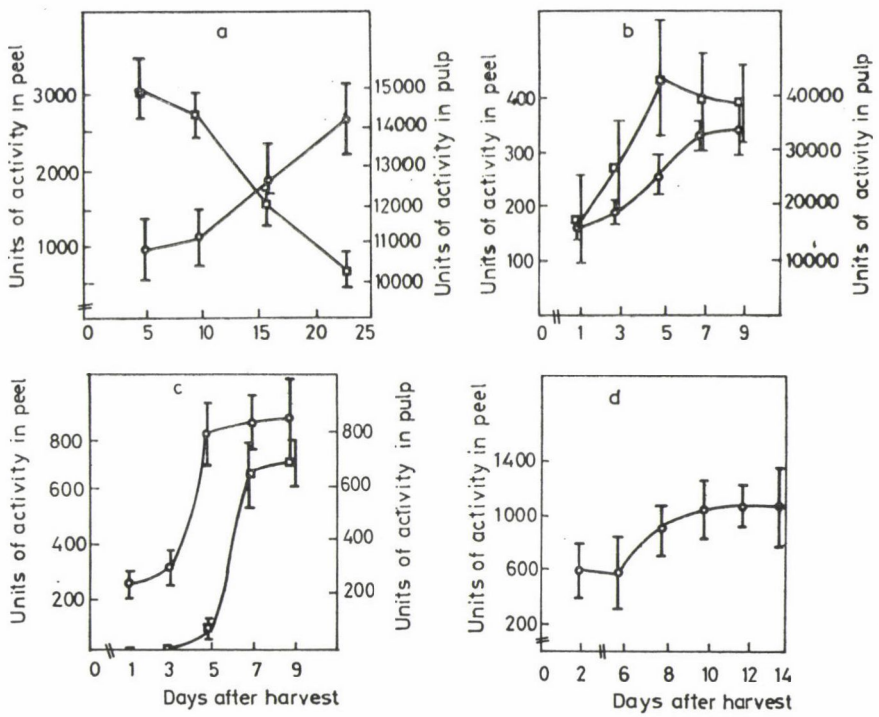

Fig. 1. PPO activities in fruits during ripening. ○—○: peel; □—□: pulp. The vertical bars represent standard error of the mean of four measurements (n = 4)

in PPO activity from unripe to the ripe stage. Sapota peel also showed an increase in the PPO activity to almost three times its initial level during ripening. In the case of sapota flesh, PPO activity could be detected in the fruits only after they had passed their climacteric. The values increased further in the later stages of ripening. In mango peel, there was a twofold increase in the PPO activity during ripening. Mango pulp tissue did not exhibit PPO activity at any stage of ripening.

2.2. Peroxidase in ripening fruits

POD activities in the peel and pulp tissues of all these fruits at different stages of ripening are given in Fig. 2 (a, b, c and d). The pattern of change in POD activity during ripening was uniform in all the fruits studied and showed a peak at or around the climacteric.

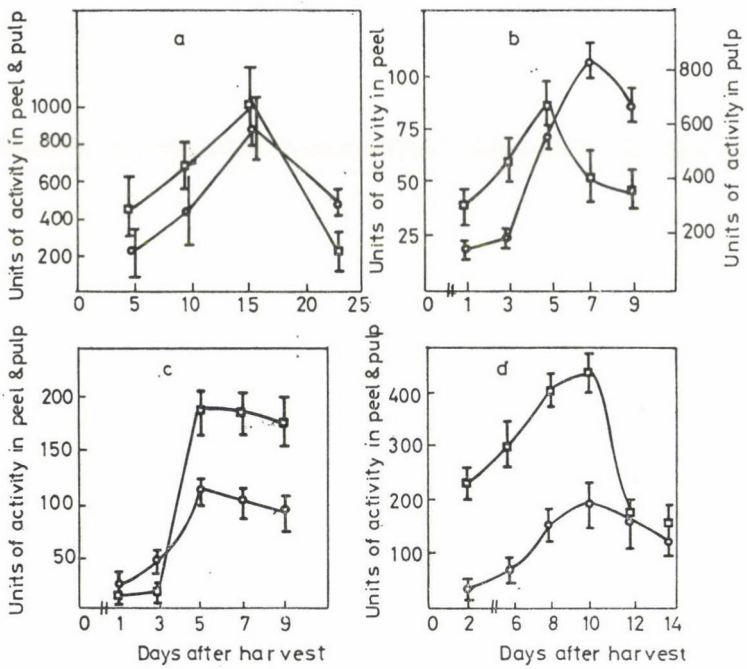

Fig. 2. POD activities in fruits during ripening. ○—○: peel; □—□: pulp. The vertical bars represent standard error of the mean of four measurements (n = 4)

2.3. PPO isoenzymes

PPO isoenzyme patterns in both peel and pulp tissues of ripening fruits are given in Fig. 3 (a, b, c and d). Figure 3a presents PPO isoenzyme pattern in the peel and pulp of unripe and ripe Royal delicious apples harvested at dif-

ferent levels of maturity. Polyacrylamide gel-electrophoretic patterns revealed a maximum of four PPO bands in the pulp and three in the peel of unripe fruits. The PPO isoenzymes in ripe fruits showed a tendency to move faster towards the anode as compared to the unripe ones. Higher anodic displacement was observed with both harvesting maturity and ripening of the fruits. There was a decrease in the intensity and number of bands in the pulp of ripe apples in comparison with unripe ones at all stages of harvesting maturity, whereas, peel isoenzymes showed a reverse tendency.

In the case of avocado (Fig. 3b) PPO bands in peel showed an increasing trend towards the anode in their movement during ripening. About 8 isoenzymes of PPO were observed in the ripe avocado peel. There were at least

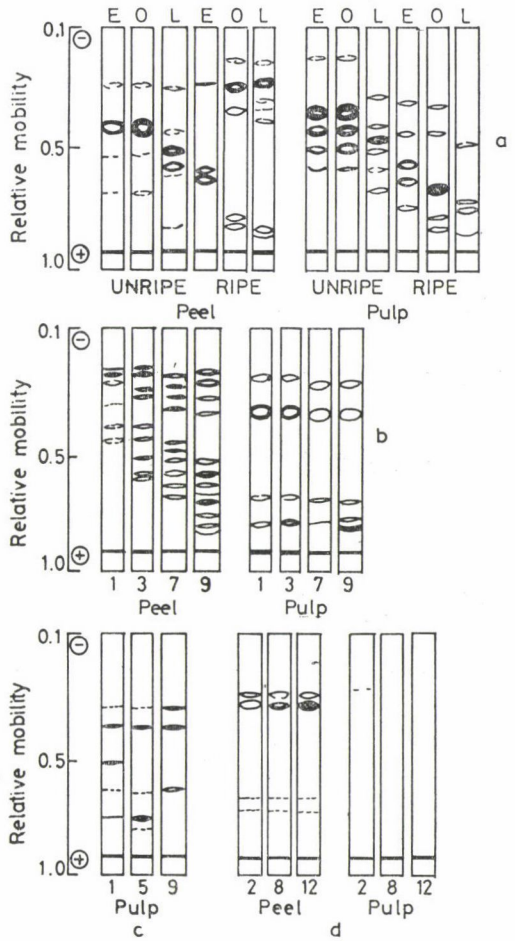

Fig. 3. PPO isoenzyme patterns in fruits during ripening. Numbering denotes days after harvest. E: early maturity; O: optimum maturity; L: late maturity; unripe: 5 days after harvest; ripe: 30, 20 and 15 days after harvest for E, O and L maturity fruits, respectively. a: apple; b: avocado; c: sapota; d: mango; —— denotes marker (bromophenol blue) front

3 bands of PPO activity in the pulp of avocado. Sapota peel and pulp revealed 4–5 PPO bands which were more intense in the ripe tissues (Fig. 3c). Mango peel PPO was separated into four isoenzyme bands two of which were most prominent and slow moving. The other two were very faint and fast moving. Mango pulp did not reveal any PPO band except for a single faint band in its unripe stage (Fig. 3d).

2.4. Peroxidase isoenzymes

The POD isoenzyme pattern in the peels and pulps of ripening fruits is shown in Fig. 4. A maximum of 4 isoenzyme bands of POD were detected in both peel and pulp tissues of apples (Fig. 4a). All the four isoenzymes showed

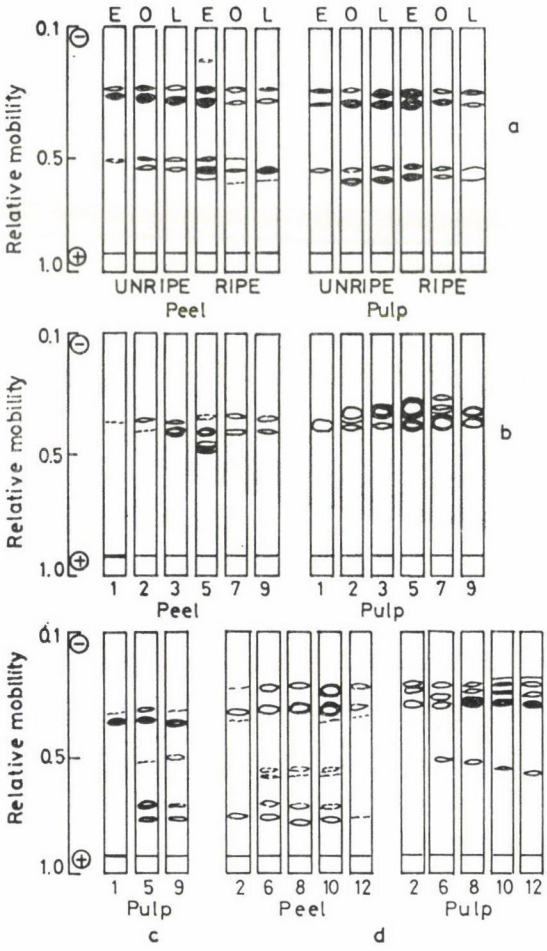

Fig. 4. POD isoenzyme pattern in fruits during ripening. a: apple; b: avocado; c: sapota; d: mango. For further legends see Fig. 3

similar R_m with respect to peel and pulp, with respect to different levels of maturity and with respect to unripe and ripe stages. The enzyme bands were observed to be more intense in the case of apples harvested at a later stage of maturity than in the earlier stages. It was noticed that the enzyme activity in the post-climacteric period was much higher than that in the pre-climacteric period in the apples harvested at early maturity. In the case of apples harvested at the late maturity stage, the enzyme activity in the post-climacteric period was lower than that in the pre-climacteric stage.

The POD isoenzymes of both peel and pulp of avocado were found to be slow moving. The bands at the climacteric stage were more prominent (Fig. 4b). There were 4 prominent bands of POD in both peel and pulp tissues of sapota which intensified during ripening (Fig. 4c). New bands of POD isoenzymes, especially fast moving ones developed during ripening in mango peel (Fig. 4b). In both mango peel and pulp POD bands were more intense and of maximum number at the climacteric stage.

3. Conclusions

The trend in activity of PPO during ripening differs in peel and pulp tissues. Generally, in all the peel tissues studied, the PPO activity increased during ripening. A similar observation has been reported for apple and banana peel (Samorodova-Bianki & Bazarova, 1971; Montgomery & Sgarbieri, 1975). In the case of pulp tissue, there was a steady decline in the PPO activity of apple during ripening and in avocado there was an increase followed by a decline. Decline in PPO activity during ripening has been reported in the flesh of tomatoes, grapes and banana (Hobson, 1967; Ferenczi & Uray, 1972; Montgomery & Sgarbieri, 1975). Interestingly, PPO activity in sapota fruit was detectable only in the post-climacteric fruit and not in the unripe fruit. But the detection of PPO isoenzyme bands in the unripe sapota extracts only upon electrophoretic separation suggests the presence of endogenous inhibitors which disappear during ripening. In the case of mango pulp, PPO activity was absent as there were no PPO bands detected. Lack of PPO enzyme or presence of PPO inhibitors may serve as a natural control for prevention of browning.

The behaviour of POD during ripening was found to be uniform and consistent with respect to the peak in activity observed in all the peel and pulp tissues during ripening. POD activity may even serve as an index of ripening in fruits. A climacteric rise in the activity of POD during ripening has been reported in the flesh of apples, banana, grapes and pear (Haard, 1977).

As far as the isoenzyme patterns are concerned PPO isoenzyme patterns were highly characteristic of individual species and variety. The relatively quicker anodic movement of the isoenzyme bands observed in apple peel and

pulp and avocado peel may be due to changes in the properties like molecular mass or dissociation phenomena or the conversion of particulate forms of the enzyme to soluble form. Cleavage of high molecular weight octamers of PPO into tetra, di and monomers were reported in apples during the course of ripening (SAMORODOVA-BIANKHI & BAZAROVA, 1971; DEMENYUK et al., 1974). Further, catechol oxidases became increasingly soluble during ripening of apples (HEIMER & MAYER, 1966) and grapes (KIDRON et al., 1978).

The enhanced POD activity observed at the climacteric stage in peel and pulp of all the fruits studied could be associated with the appearance of new isoenzyme bands as well as intensification of the already existing ones at the climacteric stage of fruit ripening. Similar observations have been reported in the case of pear, tomato and blueberry fruits during ripening (FRENKEL, 1972). However, the mobilities of POD isoenzymes unlike PPO do not appear to be affected by ripening in the fruit tissues studied.

Changes in properties like molecular mass or solubility characteristics of enzymes may bring about increased or decreased activity of the enzyme which in turn affect the degree of enzymatic darkening in post-harvest fruits. JEN and KAHLER (1974) as well as KAHN (1977) pointed out that the specific activity of PPO could be related to the discolouration of peaches and avocadoes. It is stated that these polyphenol oxidizing enzymes may play a vital role in several biochemical processes leading to fruit ripening and senescence by way of controlling phenolic levels in the fruits according to (HAARD, 1977 and MAYER & HAREL, 1979).

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INVESTIGATIONS INTO SOME ENZYME ACTIVITIES OF FINNISH WHEATS GROWN IN HUNGARY

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Three Finnish wheat varieties were grown along with the Hungarian variety Martonvásári 4 (Mv 4) on experimental parcels in Hungary. The accumulation of solids content and the activity levels of some technologically important enzymes were followed, from flowering till harvest, in the Finnish varieties Ruso and Hja 22450 (Hja) as well as in Mv 4 used as reference. Baking tests were performed with the flours of all the four varieties.

The accumulation of solids content was slower and the final kernel mass was lower in the Finnish wheats. Amylase and peptide hydrolase activities as related to kernel mass decreased while peroxidase activity increased during ripening. At the beginning of the ripening period, the decreasing order of amylase activities was Hja, Ruso and Mv 4, at full ripeness the order was reversed. With peptide hydrolase, substantial differences in activity levels were found mainly in the fully ripe state: Mv 4 showed a higher value than the other two varieties. Peroxidase activity was higher in Mv 4 throughout the ripening period.

One month after harvest amylase activities related to solids content were higher in Ruso and Hja than in Mv 4, while the third Finnish variety, Tähti, showed the lowest value. Peroxidase activity was higher in the varieties of low amylase activity and vice versa. In the baking test the water binding capacity of Mv 4 was the highest. Bread volumes obtained with the flours of higher amylase activity (Ruso and Hja) were higher. In spite of varietal differences in growth rate, kernel mass and enzyme activities, the flours of all the four wheats yielded bread of satisfactory quality.

Keywords: wheat, amylase, peptide hydrolase, peroxidase, baking properties

In a previous paper (PÁRKÁNY-GYÁRFÁS et al., 1985) an account was given on the activity levels, in some Finnish wheat samples, of enzymes important in the processing technologies. The greatest variation was obtained for amylase activity: this varied over two orders of magnitude. As this enzyme plays a decisive role in breadmaking (MERCIER & COLAS, 1967; KRUGER, 1980; FOX & MULVIHILL, 1982), it seemed expedient to know to what extent ecological factors such as climate, soil or agrotechnics might have a bearing on the enzyme activities of wheat. Therefore samples of three Finnish spring wheat varieties were grown on experimental parcels in Hungary under strictly the same conditions as a well-known Hungarian winter variety used as reference. The changes in enzyme activities were followed from flowering till harvest and were determined also in the harvested wheat and the flour obtained from it. Baking tests were carried out as well.

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1. Materials and methods

1.1. Wheat varieties

The Finnish varieties were Ruso, Hja 22450 (thereafter Hja) and Tähti; the Hungarian variety used as reference was Martonvásári 4 (thereafter Mv 4). All the varieties were sown in the spring of 1982, and grown on the experimental parcels in Tordas, of the Institute of Plant Production and Qualification, Budapest, Hungary. With the exception of Tähti, the accumulation of solids content in the kernels as well as enzyme activities were assayed at regular intervals during ripening and 30 days after harvest, before milling. With Tähti, measurements were carried out only after harvest. Analyses of the flours and baking tests were performed with the samples of all the varieties.

1.2. Sampling from flowering till harvest

Samples of ears were taken first 7 to 10 days after flowering and, subsequently, at 7-day intervals. The dates of the first samplings were: June 7 for Mv 4 and June 21 for Ruso and Hja. Mv 4 was harvested after 6 weeks (on July 12) and the Finnish varieties after 7 weeks (on August 2).

The mass of the wheat kernel was calculated weekly from the mean value of 500 kernels each obtained from 15 ears.

1.3. Determination of moisture content

During the first 3 weeks of maturation the dehulled wheat kernels were first dried at 90 °C (because of their high moisture content), then at 105 °C till reaching constant mass. In the later phases of ripening and with flours, only 105 °C was applied as drying temperature. All the measurements were carried out in triplicate.

1.4. Preparation of enzyme extracts

During the first 3 weeks of maturation the wheat samples were homogenized in a Waring blendor. From the fourth week on the preparation of the samples was carried out as described in our previous paper (PÁRKÁNY-GYÁRFÁS et al., 1985).

1.5. Determination of enzyme activities

Enzyme activities were determined using the same methods as in the paper cited above.

1.6. Milling and the baking test

Milling and baking tests were performed in the pilot equipment of the Research Institute of the Milling and Baking Industry (Budapest).

The dough was prepared from 900 g flour, 4% (w/w) yeast, 2% (w/w) salt and water according to the amount determined with the Farinograph or slightly differing from it. Kneading was performed with the kneading machine Diosna (FRG). The 30 °C dough was allowed to rise for 30 min. Three loaves of 400 g each were formed from each dough and these were subjected to further ripening at 30 °C in a raising chamber. Baking was performed at 250–260 °C in a Model C 420/90 Variomat oven (Winkler, FRG) to a bread mass of 360 g.

2. Results

2.1. Investigation of the wheat varieties from flowering till harvest

2.1.1. Development of the wheat kernel. The accumulation of solids content in the samples of the wheat varieties Mv-4, Ruso and Hja during ripening are shown in Fig. 1.

The curves show the difference in the accumulation of solids content of the Hungarian and the Finnish varieties. In the two Finnish varieties the rate and extent of the increase were nearly identical and so was the final value. The Hungarian variety had, already in the first week, a higher solids content.

2.1.2. Changes in enzyme activities. The variations observed during ripening in the activities of amylase, peptide hydrolase and peroxidase of the wheat kernel are shown in Figs. 2–4.

2.1.2.1. Amylase activity. — In the first week of ripening Mv 4 and Ruso showed considerably lower activities per kernel than Hja. In further stages of ripening the changes in amylase activity of the Hungarian and the Finnish varieties, although similar in trend, showed some minor differences. The activity of Mv 4 decreased during the first 4 weeks of ripening. The decrease was most marked between the third and fourth week (56%). By that time the kernel reached 93% of its final mass. Thereafter the decrease in activity was but slight and in the last week an increase could be observed, probably caused by rainy weather.

The changes in amylase activities of the two Finnish varieties were somewhat different during the first 4 weeks of ripening: for Hja, the values showed a nearly linear drop throughout this period, while for Ruso, a steep decrease could be observed only between the third and fourth week. A further slight decrease took place in both varieties between the fourth and fifth week. The losses of amylase activity in this period as related to the values measured on the third week amounted to about 80% and 96% for Ruso and Hja, respec-

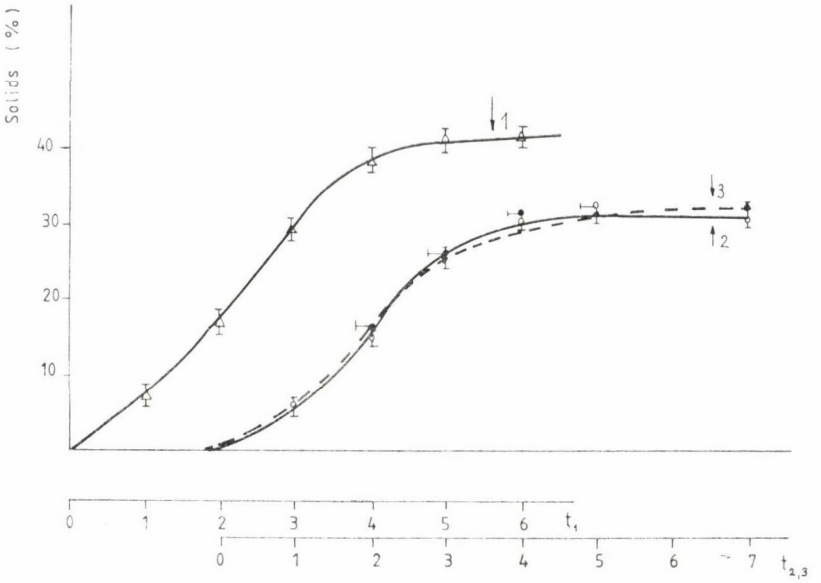

Fig. 1. Accumulation of solids content in the kernels of three wheat varieties during ripening. The varieties: Martonvásári 4 (1), Ruso (2) and Hja (3). t_1 = time from flowering till harvest for (1), $t_{2,3}$ = time from flowering till harvest for (2) and (3). 0 time = June 1 and June 14, for (1) and for (2) and (3), respectively. Harvest was on July 12 for (1) and on August 2 for (2) and (3). The vertical bars represent standard deviations. The number of replicates (n) = 3

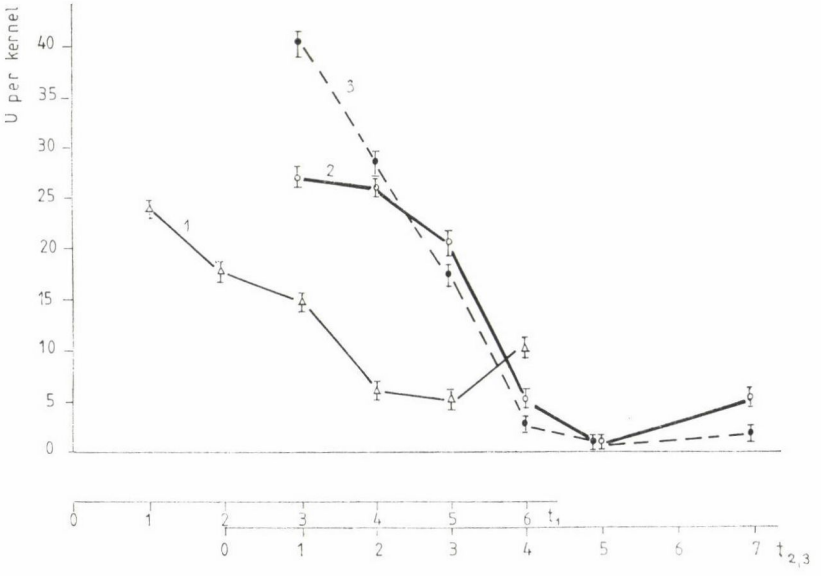

Fig. 2. Changes in amylase activity during ripening of three wheat varieties. For explanations see Fig. 1. The number of replicates (n) = 6

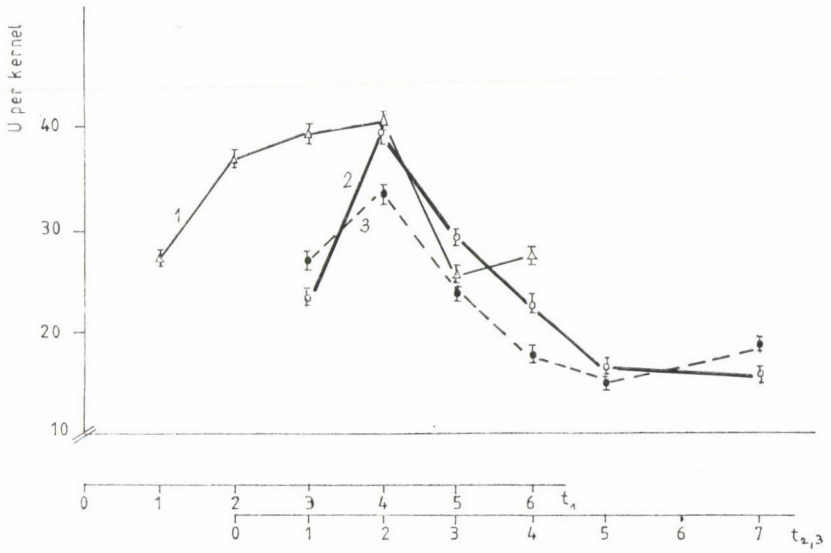

Fig. 3. Changes in peptide hydrolase activity during ripening of three wheat varieties. For explanations see Fig. 1. The number of replicates (n) = 6

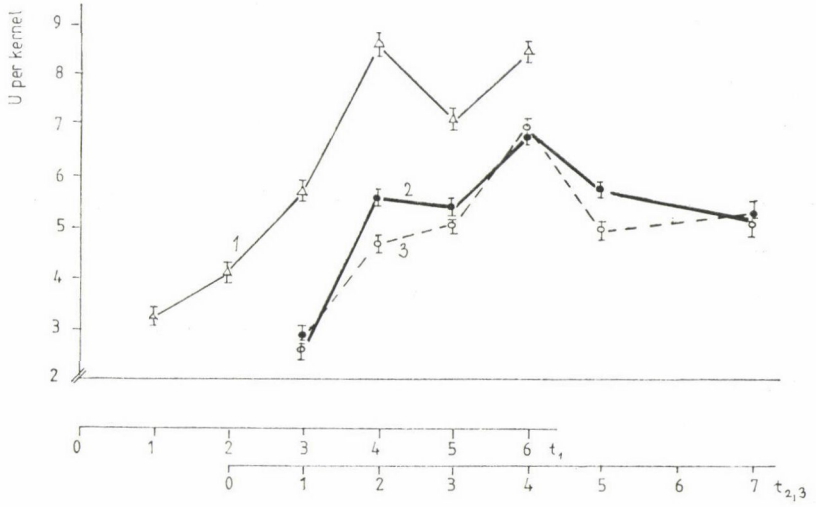

Fig. 4. Changes in peroxidase activity during ripening of three wheat varieties. For explanations see Fig. 1. The number of replicates (n) = 6

tively. By the end of the fifth week the kernels reached 92–96% of their final mass. Between the fifth and seventh week of ripening, a slight rise in activity took place. The final values were 1.7 $\pm$ 0.1 and 5.3 $\pm$ 0.2 U per kernel for Hja and Ruso, respectively. It is interesting that the order of the activities at the end of the ripening period was reversed to that measured at the beginning.

2.1.2.2. Peptide hydrolase activity. — This activity as related to kernel mass increased in Mv 4 from the first to the fourth week of ripening. A considerable decrease (37%) occurred during the fifth week. By the end of this period the kernel reached 97% of its final mass. The slight increase on the sixth week might be ascribed to the rainy weather mentioned. With the two Finnish varieties the rise in peptide hydrolase activity could be observed between the first and the second week of ripening. Between the second and the fifth week the weekly decrease in activity was, on the average, 23–25%. During the last two weeks of ripening when the kernels had reached about 96% of their final mass, variations in activity were but slight. The activities of both Finnish varieties were, at full ripeness, considerably lower than the value found in Mv 4.

2.1.2.3. Peroxidase activity. — Peroxidase activity in Mv 4 showed a steep increase between the first and the fourth week. This was followed, during the fifth week, by a decrease of 17% as related to the maximum value. The subsequent rise, probably due to the rains taking place at that period, brought about a final value similar to the maximum observed on the fifth week. The two Finnish varieties equally showed a considerable increase in peroxidase activity up to the fourth week. The rise was similar, 2.4–2.6-fold for all the three varieties. From the fourth week till harvest activities decreased in both Ruso and Hja. The decreasing period for all the three varieties started after the kernels had reached 92 to 95% of their final mass. Throughout the period of ripening activity was higher in Mv 4 than in the two Finnish varieties. These latter showed, in most cases, very similar values, including the final ones.

2.2. Enzyme activities of the harvested wheats and the respective flours

Thirty days after harvest amylase and peroxidase activities as well as moisture contents of the wheats were determined and then the samples were milled. Flour yields were as follows: 72.7%, 67.2%, 66.7% and 67.8% for the varieties Mv 4, Ruso, Hja and Tähti, respectively. Amylase and peroxidase activities of the wheats are shown in Table 1.

Amylase activities as related to solids content were, in the Finnish wheats Ruso and Hja, about 3.5- and 2.6-fold the value found in the Hungarian variety Mv 4. The Finnish variety Tähti was of the lowest amylase activity. Its value was about 1/3 of that present in Mv 4 and about 1/9 of that found in Ruso. Accordingly, the flours obtained from the wheat varieties Ruso and Hja showed the highest and the flour of Tähti the lowest amylase activity. The activity losses occurring in the milling process amounted to 71–78% of the values found in the wheats of high amylase activity and to 57% and 40%, respectively, of the values established for the wheat varieties Mv 4 and Tähti.

Peroxidase activity was lower in the two varieties of high amylase activity and nearly identical in the wheats Mv 4 and Tähti. Milling caused activity losses

Table 1

Amylase and peroxidase activities related to solids content of wheat and flour samples taken one months after harvest

Variety	Martonvásári 4				Ruso				Hja 22450				Tähti			
Activity	Amylase		Peroxidase		Amylase		Peroxidase		Amylase		Peroxidase		Amylase		Peroxidase	
	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$	$\bar{x}$	$\pm s$
Wheat	91	4	73	1.8	314	11	57	0.9	241	10	61	1.2	36	1	74	2
Flour	39	2	31	0.5	90	3	32	0.9	52	1	30	0.5	22	1	41	1.1

All the wheats were grown on experimental parcels in Hungary, in 1982.
Martonvásári 4 is a Hungarian variety used as reference, the rest are Finnish varieties.
Amylase activity is expressed in U g^{-1} and peroxidase activity in kU g^{-1}, both related to solids content. (1 kU = 1 000 U; 1 U = 10^{-3} A; A = absorbance.)

of 51–58% in the samples of Hja and Mv 4 and 43–45% in those of Ruso and Tähti.

2.3. The baking test

The water uptake of the flours as determined with the Farinograph and as observed during dough making, further the bread volumes are given in Table 2.

Table 2

Water uptake of the flours and bread volumes obtained in the baking trials

Flour of the variety	Water uptake (%)		Bread volume	
	according to farinogram	in dough preparation	(cm^3)	(%)
Martonvásári 4	63.3	63.0	1220	100
Ruso	62.1	60.0	1230	101
Hja	62.0	60.0	1260	103
Tähti	63.2	60.0	1140	93

For explanations see Table 1.

Water uptake as determined with the Farinograph was identical for the flours of Mv 4 and Tähti and 1% less for those of Ruso and Hja. Dough making confirmed the instrumentally determined value for Mv 4 only. With the other three flours a dough of suitable consistency could be obtained by using 2–3% less water than indicated by the Farinograph.

The flours of the Finnish varieties Hja and Ruso of higher amylase activity yielded bread volumes 1–3% higher than that obtained with the Hungarian variety Mv 4. (The flour of Tähti which had the lowest amylase activity gave a bread volume 7% smaller than the one obtained with the flour of Mv 4.) Crumb structure, elasticity and porosity of the breads prepared from all the four flours were satisfactory.

3. Conclusions

3.1. Development of the wheat kernel and changes in enzyme activities during ripening

The accumulation of solids in the wheat kernel was slower for the two Finnish varieties than for the Hungarian one. The average final mass of the kernels was also lower (32 mg) for the former two varieties than for Mv 4 (42 mg). This might be related to the fact that the Finnish wheats were grown under conditions to which they had not been previously adapted. For the course of solids accumulation in the kernel DONOVAN and co-workers (1977) published results similar to those presented here.

During ripening, amylase and peptide hydrolase activities decreased while peroxidase activity increased. The curves describing the processes were similar in course for the two Finnish varieties and somewhat different from those obtained for Mv 4. In the last two weeks of ripening, after the kernels had attained 90–95% of their final masses, changes in all the enzyme activities were but slight in all the samples. Similar results had been obtained in previous investigations (PÁRKÁNY-GYÁRFÁS et al., 1981). According to GORDON (1980) the decrease in amylase activity during ripening is an exponential process. The decreasing sections of the curves presented here could, however, not be fitted to exponential equations. The decrease in amylase and peptide hydrolase activities during ripening might be caused, at least partly, by the synthesis of inhibitors (PRESTON & KRUGER, 1976; WARCHALEWSKI, 1976; PACE et al., 1978; KRUGER, 1980). The increase in peroxidase activity might be related to its growth regulating function through indole acetic acid oxidation. This question will be dealt with in a separate paper.

Considering the rates of ripening and solids content accumulation as well as the final masses of the kernels and the changes in enzyme activities, it seems that ecological factors have but a limited effect on the characteristics of wheat. Similar observations have been made recently by MILLER and co-workers (1984) when growing hard wheat varieties in soft wheat areas and vice versa.

3.2. Activities in harvested wheats and their flours; evaluation of the baking tests

In our previous paper (PÁRKÁNY-GYÁRFÁS et al., 1985) dealing with some properties of Finnish wheats grown in Finland, amylase activities were found to be higher and peroxidase activities lower than the values established in the present study. It shall be noted that some wheat varieties grown on a large scale in Hungary have amylase activities in the range of some of the Finnish varieties investigated.

The ratios of amylase activities in flours and wheats were 0.42 (Mv 4), 0.29 (Ruso), 0.22 (Hja) and 0.61 (Tähti). In our previous study on Finnish wheats the corresponding range was found to be 0.34–0.45. The ratios for peroxidase were much more uniform than those obtained for amylase and were also within the range established in the previous study (0.44–0.66). Higher milling losses, especially of amylase activities of the high-amylase wheats Ruso and Hja might be explained by the fact that this enzyme is mainly located in the pericarp of the kernel (BANKS et al., 1972; KRUGER, 1972) and by the smaller kernel mass of these varieties as compared to Mv 4. The ratios obtained for peroxidase support our assumption according to which this enzyme might be located mainly in the inner parts of the kernel. The better flour yield of the Hungarian variety is obviously related to the larger size of its kernels.

Although water uptake of the Finnish wheats was below that of Mv 4, bread volume in the baking tests varied more or less in parallel to amylase activity. In our previous study the relative bread volumes obtained with Finnish flours of amylase activities of 746, 178 and 17 U g^{-1} were 85, 93 and 100. Although the two series of baking tests have been performed in different ways, only the flour of extremely high amylase activity (746 U g^{-1}) yielded a considerably smaller bread volume than the rest. The differences in bread volumes obtained with flours of very similar amylase activity (e.g. 17 and 22 U g^{-1}) in the samples of the two series might be accounted for by different ratios of the two main groups of amylase isoenzymes in the respective wheats. The "green" type amylases and those similar to the "germinated" amylases but formed during growth and ripening have been reported to differ in some characteristics important from the aspect of breadmaking (e.g. pH optimum and heat stability, KRUGER, 1980). It cannot be precluded that ecological factors might affect the ratio of these two groups of isoenzymes in wheats without substantially altering the overall amylase activity.

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DISINFESTATION OF COMMERCIALLY PACKED DATES BY A COMBINATION TREATMENT

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No significant difference has been found between male and female *Ephestia cautella* larvae irradiated with low disinfestation doses of gamma radiation with regard to their ability to damage dry dates.

The longevity of *E. cautella* larvae detected in dry date packages was significantly shorter when these packages were treated with 0.7 kGy at 25 °C, exposed to 40 °C for 48 h, then transferred to 25 °C and stored for 15 days as compared to the irradiated control which was kept at 25 °C only.

Treatment with 0.35 kGy and heat also brought about some decrease in longevity. On the other hand, the dry date damaging ability of irradiated *E. cautella* larvae was significantly lower when last instar larvae treated with 0.7 kGy gamma radiation were exposed to 40 °C for 72 h.

Thus combination treatment could lead to a decrease in the feeding ability of the highly date damaging stage of *E. cautella*, and to a shorter period of time to cause 100% mortality which will comply with conventional quarantine restrictions. Other types of treatments also showed the superiority of combination treatments for date disinfestation.

Keywords: combined treatment, irradiation, packed dates, disinfestation

Insect disinfestation of dry dates by low doses of gamma radiation from a ^{60}Co source (WHO, 1981) usually leads to a lifespan prolongation of last instar *Ephestia cautella* larvae (AHMED, 1981) which is one of the main pests of stored dates in Iraq (HUSSAIN, 1974). In this respect it is of interest to assess the percentage of date infestation that might be brought about by irradiated insect developmental stages particularly the highly radioresistant *E. cautella* last instar larvae (AHMED et al., 1972). Since combination treatment of radiation and heat shocks might synergistically aggravate the genetic (HASHIM-AHMED, 1965, 1967) as well as the non-genetic physiological or killing effects (BALDWIN & NARRAWAY, 1957) of insects irradiated before heating, it is worthwhile to utilize this phenomenon to decrease the longevity of the irradiated larvae and minimize their ability to damage dry dates. Therefore the present experiments have been carried out for the following purposes:

— To find out if there is a difference in the date damaging ability between irradiated male and female *E. cautella* larvae;

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— to measure some biological effects of postirradiation exposure of *E. cautella* larvae to elevated temperature (40 °C);

— insect disinfestation by combination treatments (gamma irradiation and heating at 40 °C) of commercially packed dry dates, Zahdi variety.

1. Materials and methods

1.1. Difference in the date damaging ability of irradiated E. cautella *male and female larvae*

Details of the method used are mentioned in the early reference (AHMED et al., 1985) except that the added treated or untreated *E. cautella* last instar larvae to each replicate were sexed depending on the conspicuous tests easily seen on the dorsal side of the abdominal segments of the larvae to assess the dry date damaging ability of males and females, separately. (See also para. 1.2. below for more details.)

1.2. Effect of combination treatment on the date damaging ability of E. cautella *larvae*

Fifty uninfested non-irradiated date fruits (Zahdi variety) were put in a beaker (1000 cm^3) plus 30 g *E. cautella* last instar larvae (without sexing) in four replicates for each of the following treatments: 0 kGy (control, untreated larvae) at 25 °C; 0 kGy and kept for 72 h at 40 °C then transferred to 25 °C; 0.7 kGy of gamma irradiation at 25 °C; and 0.7 kGy at 25 °C and exposed for 72 h to 40 °C then transferred to 25 °C. The four replicates set up of each treatment (16 beakers) were kept for about 45 days and the rate of infestation was recorded according to the method previously followed (AHMED et al., 1985).

1.3. Disinfestation of packed dates by a combination treatment

Heavily infested dry dates, prepared as reported in the early literature (AHMED et al., 1985) with minor alterations, have been packed in 96 window carton lunch boxes automatically sealed with laminated cellophane film (CBs). They were then distributed in 6 standard carton boxes (SCBs) each containing 16 CBs. Each SCB with 16 CBs has been subjected to either of the following treatments:

— 25 °C;
— 40 °C for 48 h then transferred to 25 °C;
— 0.35 kGy gamma irradiation at 25 °C;
— 0.35 kGy at 25 °C and 40 °C for 48 h then transferred to 25 °C;
— 0.7 kGy 25 °C;
— 0.7 kGy at 25 °C and 40 °C for 48 h then kept at 25 °C.

Approximately every 15 days, dates in 4 CBs taken at random out of each SCB were carefully examined and the rate of date infestation was determined as in 1.2. Since reinfestation was detected in the CBs taken from the 6 SCBs as the development and genetic tests (AHMED et al., 1982) have shown in the last 3 out of 4 examinations, the results of the first examination only (after 15 days of storage) are detailed below.

2. Results and discussion

2.1. Damaging ability of E. cautella *male and female larvae*

Analysis of variance was followed to test the significance in date infestation percentages brought about by irradiated males or females of *E. cautella* last instar larvae (STEEL & TORRIE, 1960). Duncan multiple range tests ($P \leq 0.05$) showed that using a dose of 0, 0.35, 0.70 or 1.05 kGy, no significant difference in the date infestation rates was noticed when the damaging effect of male or female was separately examined (Table 1). This is in contrast to other insects where the adult females are more radioresistant than the heterogametic males (NOTHEL, 1968). This fact possibly demonstrates that sex ratio distortion in *E. cautella* in favour of the homogametic males (AHMED et al., 1972), in case of utilizing sterile insect technique for control or eradication purposes, does not effect any differential damaging ability of importance as far as dry dates are concerned.

Table 1

Effects of gamma irradiation on the date damaging ability of male or female last instar larvae of the fig moth, Ephestia cautella
(50 sound dates + male or female larvae per treatment, average of 3 replicates examined 45 days after treatment)

Larvae added	Dose (kGy)	Average of date fruit damaged[a] (%)		Remarks
Males	0[b]	33.0	AB	Untreated larvae
	0.35	31.33	AB	
	0.70	10.0	C	
	1.05	7.3	C	
Females	0	38.0	A	Untreated larvae
	0.35	26.67	B	
	0.70	11.33	C	
	1.05	10.67	C	
Nil[c]	0	0.0	C	No larvae added

[a] Means followed by the same capital letter in a column are not significantly different at the 5% level, by Duncan's multiple range test
[b] Only 2 replicates
[c] 50 sound dates without larvae

2.2. *Effect of combination treatment on the date damaging ability of* E. cautella *larvae*

The damaging ability of irradiated *E. cautella* larvae was significantly lower (at $P \leq 0.05$) when last instar larvae treated with 0.7 kGy gamma radiation were exposed to 40 °C for 72 h (Table 2). Such result is in agreement with the fact that postirradiation exposure of insects to high temperature usually modifies and increases the genetic as well as the non-genetic effect of irradiation (BALDWIN & NARRAWAY, 1957; AHMED, 1981; AHMED et al., 1981) which can be utilized in disinfestation of different commercial date packages (DPs).

Table 2

The effect of gamma irradiation and combination treatment on the date damaging ability of last instar larvae of E. cautella[a]

Type of treatment	No. of dates infested	No. of dates uninfested	Infested dates (%)	*t* value	Probability level (P)
25 °C for 72 h	185	15	92.5	2.09	≥0.05
40 °C for 72 h	149	51	74.5		
0.70 kGy + 25 °C	66	134	33.0	2.34	≤0.05
0.70 kGy + 40 °C	48	152	24.0		

[a] Using 50 sound uninfested dates (Zahdi variety) plus 30 treated *Ephestia* larvae in a beaker (1000 cm^3) in 4 replicates for each dose and examined 45 days after treatment

2.3. *Disinfestation of packed dates by combination treatments*

Based on both the previous observations (AHMED et al., 1981) and the above-mentioned results, disinfestation of DPs was carried out by using combination treatment of radiation and mild heat (40 °C). The results shown in Table 3 indicate that the longevity of *E. cautella* larvae detected in the DPs was significantly shorter when the DPs were treated with 0.7 kGy, exposed to 40 °C for 48 h, then transferred to 25 °C and stored for 15 days as compared to the irradiated control which was kept at 25 °C only.

Table 3

Longevity of Ephestia cautella *larvae found in the packed infested dates, 15 days after exposure to radiation or combination treatments*

Dose (kGy)	Longevity day: Packages kept at 25 °C $\bar{x}$ ±s	Longevity day: Packages kept at 40 °C for 48 h then at 25 °C $\bar{x}$ ±s	*t* value	Probability level (P)
0.35	20.6 ± 8.65	16.4 ± 9.89	1.62	≥0.05
0.70	10.1 ± 4.24	6.7 ± 3.49	3.57	≤0.01

Average value ($\bar{x}$) and standard deviation (±s)

Treatment with 0.35 kGy and heat also brought about some decrease in larval longevity. Thus combination treatment could lead to a decrease in the feeding ability of the highly date damaging stage of *E. cautella*, and to a shorter period of time to cause 100% mortality which will comply with conventional quarantine restrictions (CORNWELL, 1966; BURDITT, 1982; GIDDINGS, 1983). Other types of treatments also showed the superiority of combination treatments for date disinfestation which might be of commercial importance (AHMED et al., 1981).

In this respect heat gain from solar radiation can be profitably exploited for example by ventilating only in a limited period during the day or by other availabe methods to maintain the desired level of mild temperature (40 °C) for a while in the internal environment of a storage building in a hot season in Iraq.

*

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DETERMINATION OF THE CYSTINE CONTENT OF FOODS AND FEEDS BY MERCAPTO-ETHANE-SULFONIC ACID HYDROLYSIS

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The authors reported in an earlier paper (CSAPÓ, 1983) that among all the hydrolytic methods the triptophan content was highest when the protein was hydrolyzed with mercapto-ethane-sulfonic acid. However, it is an inherent error of the method that cystine cannot be determined by it because of the reduction of cystine to cysteine and the presumed thioether binding between cysteine and the sulfhydryl group of mercapto-ethane-sulfonic acid (MES-OH). The present paper reports on the determination in foods and feeds by ion-exchange column chromatography of cysteine and of cystine as established through cysteine MES-OH hydrolysis. Using the cysteine prepared from cystine and MES-OH as standard, the cystine contents of different samples are well comparable with the results of determinations after hydrolysis with 6 mol hydrochloric acid subsequent to oxidation according to MOORE and STEIN (1963) with 6 mol hydrochloric acid or to LIU and CHANG (1971) with 3 mol para-toluene-sulfonic acid or to HIRS (1956) with performic acid. When determining the cysteine in an amino acid analyzer the cystine added to the samples can be recovered at an efficiency above 90%.

Keywords: reduction of cystine, cysteine determination, protein hydrolysis with MES-OH, thioether

To determine the tryptophan content of protein PENKE and co-workers (1974) hydrolyzed it with MES-OH. The tryptophan content was recovered after 22 h of hydrolysis as an average of 5 analyses at 94.5%. It is noted, however, by the authors that the method was not used for the determination of tryptophan in foods. The analyses carried out in 1983 by the authors of the present paper proved wholly the findings of the above authors (CSAPÓ, 1983). Of all methods of hydrolysis tested the highest values on tryptophan were obtained by using MES-OH for hydrolysis. Carrying out the hydrolysis at the optimal temperature for sulfonic acids, at 125 °C, the values obtained for all amino acids, except for cystine, were satisfactory. Due to the reducing character of the medium the methionine content of the samples was substantially higher than with any of the other hydrolyses. It is a serious disadvantage of the method, however, that probably due to the reduction of cystine and the thioether binding occurring between the sulfhydryl group of MES-OH and cysteine it cannot be used for the determination of cystine. The formation of hydrogen sulfide during the reaction is an indirect proof of the thioether binding. The hydrogen sulfide formed does not permit of the establishment of the amount of

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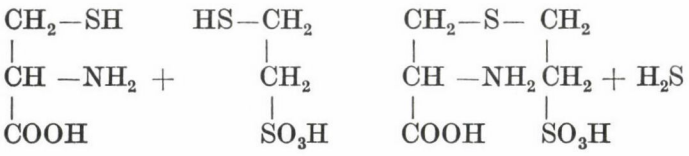
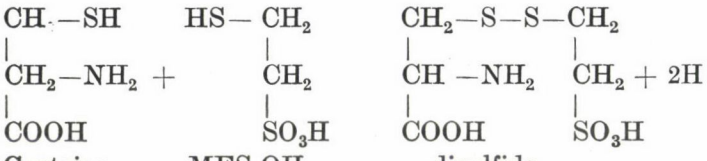
cysteine present in the thioether binding, because it may originate from the breaking down of MES-OH during hydrolysis.

The equation of the reaction is presumed to be as follows:



Cysteine MES-OH 2-amino-3-/2-sulfoethyl-thio/-propionic acid

According to LIVERMORE and MUEKE (1954) cysteine may form with other sulfhydryl compounds present mixed disulfides. Accordingly a possible disulfide formation occurs by the following reaction:



Although thiols are very sensitive to oxidation and may be dehydrogenized to disulfide even by a mild oxidative effect, the reaction leading to disulfide formation is not probable in the reductive medium of MES-OH. The first reaction, where thio-ether binding occurs between two sulfhydryl groups, is much more probable.

Experiments were carried out to find out whether the 2-amino-3-(2-sulfoethyl-thio) propionic acid (hereinafter thioether) obtained by the above reaction can be separated from the rest of the amino acids by ion exchange column chromatography and added to the amount of cysteine obtained by reduction permitted of determining the cystine content of the samples. Or the cysteine and the perhaps ninhydrin-positive compound obtained is washed in one of the amino acid peaks thereby falsifying the result of determination. The results of the experiments are given in the following.

1. Materials and methods

1.1. Materials

A sample of low and another of high cystine content were examined. Powdered milk was used as a sample of low cystine content. This was obtained by immediate lyophilization at −50 °C of milk fresh from Holstein-Friesian cows (Type OE-950 Labor MIM, Hungary). The raw protein content of the

powdered milk was 35.4% as established on the Kjel-Foss rapid nitrogen analyzer (Kjel-Foss 16200, Foss Electric, Denmark). Feather meal was selected as a sample of high cystine content. The feather was obtained by plucking white boiler hens. Care was taken to keep the natural proportion of down to coverts. The feather was first dried in a drying oven for 24 h at 60 °C and cut up with a pair of scissors into 2–3 mm pieces and finally milled in an attrition mill (DFH-48, Janke-Kunkel, FRG). The raw protein content of the feather meal was 90.8%. The protein content of the milk powder was calculated as $N(\%) \times \times 6.38$, while that of the feather meal as $N(\%) \times 6.25$.

1.2. Hydrolysis and processing of the hydrolysate

After defatting the samples 50 mg each were weighed into 10 cm^3 ampoulles previously washed with chromic sulfate. To each ampoulle 10 cm^3 3 $mol\,l^{-1}$ mercapto-ethane-sulfonic acid (MES-OH) was added (Pierce, Product number: 25555). The ampoulles were kept for 24 h at 125 °C as suggested by LIU and CHANG (1971). After cooling the ampoulles were broken and their pH was set at 2.2 with 4 mol sodium hydroxide while keeping the ampoulles in salt–ice cooling mixture to prevent their temperature from rising above 30 °C. The hydrolysates were then washed into 50 cm^3 volumetric flasks with citrate buffer of pH 2.2. After making up to the mark the hydrolysates were filtered on Filtrak 388 filter paper then kept at −25 °C in teflon vessels till applied to the automatic amino acid analyzer (LKB 4101, LKB Biochrom Ltd., UK).

1.3. Analysis

The amino acids were determined in an LKB 4101 type automatic amino acid analyzer using Merck amino acid calibration standard. Otherwise the analyses were carried out as described by CSAPÓ (1983). The cysteine standard was prepared and processed according to chapter 1.2 by weighing 50 mg cystine into 10 cm^3 ampoulles and keeping at 125 °C for 24 h with 5 cm^3 3 mol MES-OH. The cysteine peak thus obtained was used as equivalent to 50 mg cystine appropriately diluted.

1.4. Evaluation of the chromatograms

The quantitative evaluation of the individual amino acids was carried out by comparing the area under the peak in the chromatogram of the samples to the area under the peak of the standard amino acid. To the quantitative determination of cysteine the standard as prepared according to 1.2 was used.

1.5. Statistical analysis

Mean values of results, standard deviations, comparison of mean values, one variable variance analysis were calculated on the pocket computer Type PTK-1096 (Híradástechnikai Szövetkezet Budapest, Hungary). The homogeneity of the results compared was tested by Bartlett's test.

2. Results

In Fig. 1 the chromatogram of MES-OH kept at 125 °C for 24 h, is shown. In the chromatogram directly behind the front appears the ninhydrin-positive peak the absorption of which at 440 nm is the multiple of the absorption at

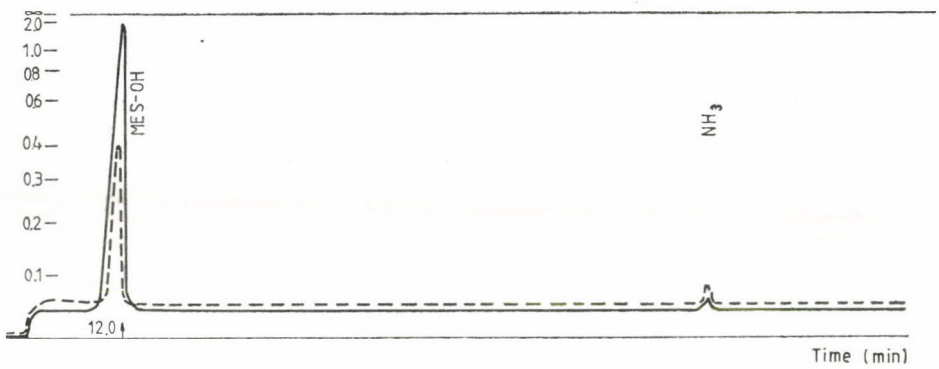


Fig. 1. Chromatogram of mercapto-ethane-sulfonic acid (MES-OH)

570 nm. At the location of ammonia a ninhydrin-positive peak, hardly evaluable, was found. (Abbreviations used in the figures: MES-OH – mercapto-ethane-sulfonic acid; Asp – aspartic acid; Thr – threonine; Ser – serine; Glu – glutamic acid; Pro – proline; Gly – glycine; Ala – alanine; Val – valine; Met – methionine; Ile – isoleucine; Tyr – tyrosine; Leu – leucine; Phe – phenylalanine; NH_3 – ammonia; Lys – lysine; His – histidine; Arg – arginine.)

Figure 2 shows the chromatogram of 250 times diluted 50 mg cystine treated with 5 cm^3 MES-OH for 24 h at 125 °C. The peak of MES-OH appears after the commencement of analysis in the place of cysteic acid. In the 44th minute cysteine is absorbed near proline in the direction of glycine. Ammonia is present in minute amount but can be well evaluated. At the buffer–ninhydrin ratio as applied, the light absorption of cysteine at 440 nm wavelength is 65.4% higher than at 570 nm (established on the basis of regions under the peaks).

In Fig. 3 MES-OH, cysteine and the 50 mg cystine, added to the hydrolysate prior to applying it to the analyzer, are shown. The cysteine peak appears

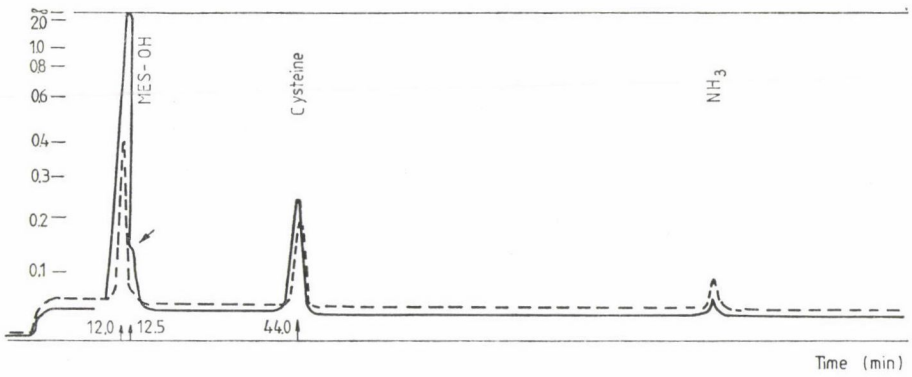


Fig. 2. Chromatogram of MES-OH and cystine, treated for 24 h at 125 °C

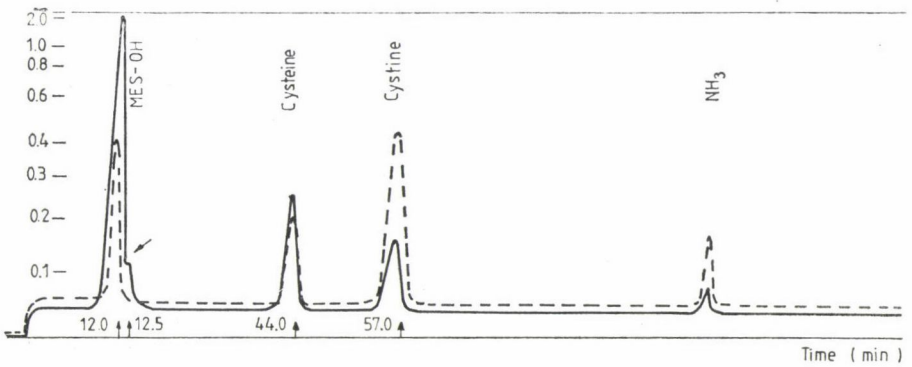


Fig. 3. Chromatogram of MES-OH and cysteine and cystine added after hydrolysis

in the 44th min of the analysis and the cystine peak reaches its maximum in the 57th min. Ammonia can be well evaluated in the chromatogram. In Figs. 2 and 3 a shoulder peak is well visible behind the MES-OH peak marked with an arrow in the chromatogram, but it cannot be used for quantitative determination because it is partly merged into the MES-OH peak. Since this peak cannot be identified in Fig. 1 it is assumed to belong to thioether formed in comparison to cysteine in a very low amount.

In Fig. 4 appears the feather meal hydrolysate obtained with 6 mol HCl at 110 °C for 24 h. It is apparent in the chromatogram that feather meal contains a high amount of cystine, while very little methionine, lysine and hystidine. The peaks of MES-OH and of cysteine are naturally missing from this chromatogram.

Figure 5 shows the chromatogram of feather meal hydrolysate obtained by hydrolysis with 3 mol MES-OH for 24 h at 125 °C. Peaks belonging to acidic amino acids appear behind the peak of MES-OH. Under the buffer sys-

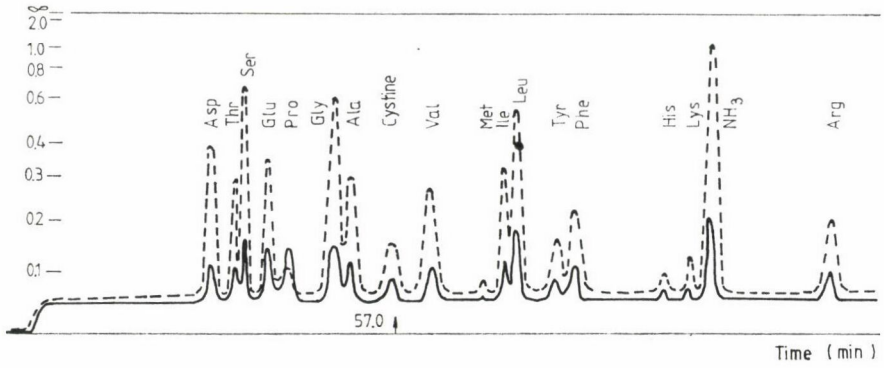


Fig. 4. Chromatogram of feather meal hydrolyzed with 6 mol HCl

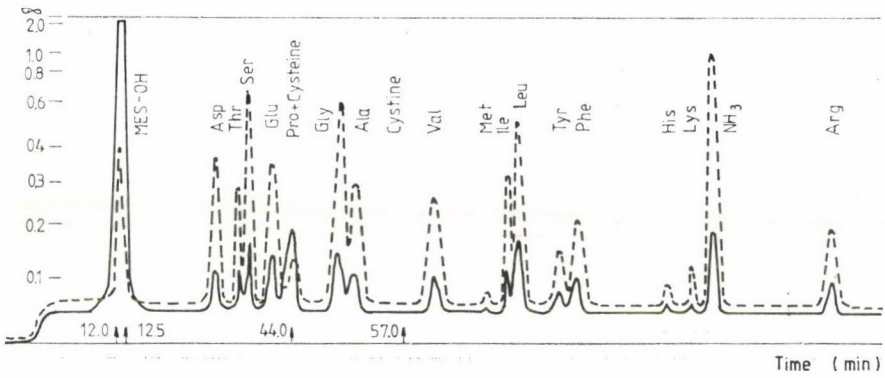


Fig. 5. Chromatogram of feather meal hydrolyzed with 3 mol MES-OH

tem applied, the peaks of proline and of cysteine are merged and cannot be evaluated separately. The cystine peak is missing between the peaks of alanine and valine. The rest of amino acids is comparable to the peaks obtained by hydrolysis with 6 mol HCl.

The chromatogram seen in Fig. 6 was obtained as follows. The temperature of the ion exchange column was set at 30 °C. The pH of buffer I was set at 3.35 instead of 3.25. The alcohol concentration was increased 1.25-fold. Thereby it was achieved that the proline peak was merged into that of glutamic acid and in the place of proline appeared the peak of cysteine. The merging of proline into glutamic acid was checked by added proline.

This method did not improve the separation of the peaks of MES-OH and that presumed to belong to thioether. The next step was the determination of the amino acid composition of the milk powder by MES-OH hydrolysis. The cystine peak completely disappeared from the chromatogram and it was not possible to detect cysteine because it was formed in a very low quantity.

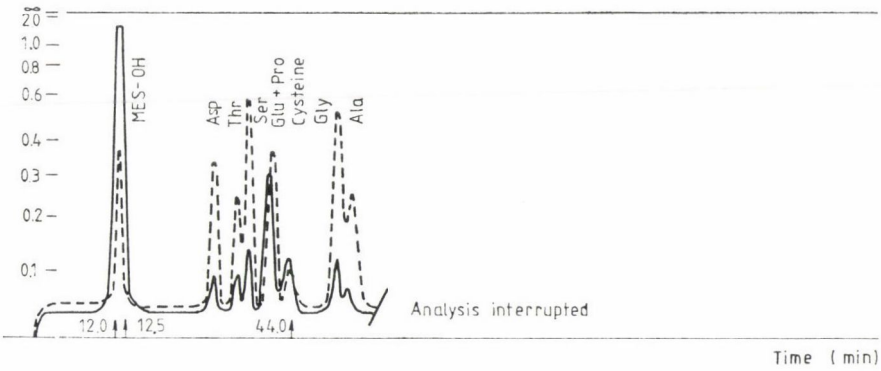


Fig. 6. Determination of cysteine.

The cysteine content of feather meal as obtained by hydrolysis with 6 mol HCl or 3 mol MES-OH in 5 parallel measurements is presented in Table 1. Table 2 contains the one variable variance analysis. Results of experiments into the recovery of added cystine, are shown in Table 3. The cysteine standard as obtained according to para 1.2 was used in quantitative determinations as equivalent to the cystine used in the preparation of cysteine and other conversions (thioether, disulfide) and breaking down were left out of consideration.

Table 1

Cystine content of white hen's feather meal obtained by hydrolysis with 6 mol HCl or 3 mol MES-OH

Method of hydrolysis	Cystine content (g amino acid per 100 g feather)	(g amino acid per 100 g protein)
6 mol HCl		
mean value	6.33	7.50
standard deviation	0.19	0.23
standard deviation as % of mean	3.00	3.10
3 mol MES-OH		
mean value	6.13	7.26
standard deviation	0.29	0.35
standard deviation as % of mean	4.73	4.82

Table 2

One variable variance analysis of the results of cystine determination by hydrolysis with 6 mol HCl or 3 mol MES-OH

Degree of freedom		F value		Probability level
of numerator	of denominator	calculated	tabulated	
1	8	1.41	3.46	P = 10%

Table 3

Recovery of added cystine

Added cystine (g amino acid per 100 g sample)	Method of hydrolysis	
	6 mol HCl	3 mol MES-OH
feather meal	6.33 (100%)	6.13 (100%)
99 mg feather meal 1 mg cystine	7.28 (99%)	6.92 (97%)
98 mg feather meal 2 mg cystine	8.19 (98%)	7.84 (96%)
97 mg feather meal 3 mg cystine	9.12 (98%)	8.72 (96%)
95 mg feather meal 5 mg cystine	11.10 (98%)	10.58 (95%)
90 mg feather meal 10 mg cystine	15.99 (98%)	14.63 (91%)

Values in brackets represent recovery percentage related to the theoretical value

3. Conclusions

It is clearly visible in Figs. 1 to 6 that in consequence of hydrolysis with MES-OH the peak belonging to MES-OH appears in the front and its absorption at 440 nm wavelength is the multiple of that as measured at 570 nm. When cystine is added to MES-OH, after hydrolysis the cysteine formed in the reaction between MES-OH and cystine appears in the place of proline but somewhat nearer to glycine. The light absorption of cysteine at 440 nm is 65.4% higher than at 570 nm under the buffer-ninhydrin ratio as used in the experiments.

As seen in Fig. 3 cystine appears in the chromatogram substantially later than cysteine and the character of the peak and its light absorption differs significantly from that of cysteine.

In Figs. 2 and 3 appears a ninhydrin-positive peak partly merged into the MES-OH peak and not suitable to quantitative evaluation but belonging probably to the thioether formed. On comparing the chromatograms of feather meal as obtained after hydrolysis with 6 mol HCl or 3 mol MES-OH the cystine peak disappears after hydrolysis with 3 mol MES-OH and the cysteine formed substantially increases the proline peak. Thus it may be concluded that by normal chromatography resulting in nearly optimal separation of all amino acids, cysteine cannot be separated from proline. By increasing the pH and alcohol content of buffer I and simultaneously reducing the temperature of the column the cysteine becomes separable from proline and can be quantitatively evaluated in the chromatogram. However, at the same time glutamic acid and proline become inevaluable and glycine becomes significantly more difficult to separate from alanine.

Results in Table 1 prove that the difference in the cystine content of feather meal, as determined in the form of cystine after hydrolysis with 6 mol HCl or in the form of cysteine after hydrolysis with 3 mol MES-OH, is non-significant although the mean of cysteine measurements is somewhat lower than that of cystine measurements. Data in Table 3 show that added cystine

determined as cysteine is recovered with an efficiency above 90%. The cysteine standard used in the measurements was prepared from cystine and considered equivalent to cystine, not accounting for other conversions or decompositions. In spite of the results obtained it is not advised to apply MES-OH hydrolysis in serial cystine determinations because of the methodical difficulties involved. If we wanted to determine all the amino acids from the same MES-OH hydrolysate then we would have to carry out two separate analyses, because in the altered buffer I glutamic acid and proline could not be determined while the glycine and alanine determinations would be unreliable. A further disadvantage of the method lies in the fact that it can be used only with proteins of high cystine content. In substances of low cystine content as the majority of foods and feeds, it is very difficult to determine cystine.

Summing up the results it can be established that it is possible to determine cystine in MES-OH hydrolysates, however, because of methodical difficulties this is not advised. It is more expedient to oxidise the cystine with performic acid to cysteine and in accelerated application determine it on the amino acid analyzer (CSAPÓ, 1982) or according to HOLZ (1981) photometrically.

A further conclusion of our experiments is that in MES-OH hydrolysis the cysteine formed from cystine appears in the chromatogram in the place of proline and this, particularly in the case of proteins of high cystine content, falsifies the result of proline determination.

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CORRELATION BETWEEN SENSORY AND GAS-CHROMATOGRAPHIC MEASUREMENTS ON GRAPEFRUIT JUICE VOLATILES

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The relationships between sensory responses and gas-chromatographic data were investigated in grapefruit juice by simple linear regression analysis in conjunction with odour threshold concentrations for determining volatile compounds important to grapefruit juice flavour. According to the results, there are some compounds such as methyl butyrate, ethyl butyrate, limonene, decanal and nootkatone which contribute significantly to pleasantness of grapefruit flavour, while others such as trans- and cis-epoxydihydrolinalool and α-terpineol contribute to unpleasantness of flavour when their concentrations increase during storage of the juice.

Keywords: volatiles of grapefruit, sensory evaluation, gas chromatography, grapefruit juice

The flavour of any food is largely perceived as a result of the release of volatile odorous components, usually present in only trace amount in food, into the air in the mouth and thence to the olfactory epithelium in the nose. There have been a great desire and a continuous effort to reduce subjective flavour responses, and gas liquid chromatography (GLC) is one of the general approaches used in attempts to obtain objective measurements of flavour quality (JENNINGS, 1977; POWERS, 1981). This relation can be useful not only in quality control, but also to determine which compounds are important to the flavour if it is used in conjunction with procedures for determining flavour thresholds.

AHMED and co-workers (1978) reported the contribution to orange flavour of a few volatile constituents of the juice using only threshold values. However the contribution of most of the authors are uncertain because, by this method, it is impossible to evaluate the masking, additive, enhancing or synergistic effects when various compounds are in combination.

The present paper reports on experiments to find models for relationships between sensory response and GLC which are exact enough for determining volatile compounds, important to grapefruit juice flavour.

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1. Materials and methods

1.1. Materials

Canned single-strength grapefruit (Marsh and Ruby Red varieties) juice samples were picked up at a commercial plant in Cuba at weekly intervals during four months so that 16 fresh samples were obtained for the purposes of this study. Another 8 samples were obtained from samples stored at 20, 26 and 30 °C, respectively, for several weeks.

Chemicals used were obtained from BDH Chemicals Ltd., Merck and others donated by the Essential Oils Laboratory from CENIC. The purity of these compounds exceeded 99% with the following exceptions: limonene (96%), myrcene (90%), citral (mixture of 67% geranial and 33% neral), epoxydihydrolinalool (mixture of 75% trans-isomer and 25% cis-isomer), nootkatone (96%), α-terpineol (95%) and geraniol (94%). To prepare an aqueous solution of each compound, the reagents were first dissolved in a small amount of absolute ethanol, since some of them were relatively insoluble in water. The water used in this study was double glass-distilled. Wide-mouthed amber glass bottles (60 cm^3) that were individually capped were used in the sensory tests.

1.2. Aroma isolation and GLC analysis

The isolation and concentration of grapefruit volatiles for GLC were performed by distillation of 500 cm^3 of juice in a vacuum rotary evaporator (Rotadest, Hungary) and the distillate (400 cm^3) was trapped in one cold trap cooled with ice and two traps cooled with liquid nitrogen. The bath temperature was 40 °C and the pressure was 1.33 kPa. The volatile compounds were extracted from the distillate and the liquids in the traps with double-distilled diethyl ether (3 times with 60 cm^3 each) prior to saturation of the distillate with sodium chloride. Then, the extract was concentrated by careful distillation to 1 cm^3. The resulting concentrate maintained the characteristics of the original aroma of each sample when it was diluted to the original volume.

The GLC was carried out on a Chrom 4 (Czechoslovakia) instrument equipped with a dual flame ionization detector. The separation was performed on 0.4 cm (o.d.) × 2.5 m stainless steel columns packed with 80 per 100 mesh Chromosorb W coated with 10% Carbowax 20 M (BDH Ltd.). Argon at a flow-rate of 30 $cm^3\ min^{-1}$ was the carrier gas and the oven temperature was 70—210 °C at 6 °C min^{-1}, then isothermic at 210 °C for 15 min. The injector temperature was 200 °C.

Peak identification was made by comparison of retention data on different chromatographic columns and peak enrichment technique with compounds reported in previous papers (Kirchner et al., 1953, Kirchner & Miller, 1953; Moshonas & Shaw, 1971; Pino et al., 1985; Pino, 1984).

For quantitative analyses the peak areas were graphically measured and the results were expressed as p.p.b. on the basis of a previously described internal standard method (PINO, 1980) which considers the factor response and extraction coefficient of the identified compounds. For regression analysis the quantitative data were expressed as percentage of the total areas of volatile compounds. Chromatographic runs were made in duplicate.

1.3. Sensory evaluation

Odour threshold concentrations were determined using the single stimulus difference test employed by SIEK and co-workers (1969) and LANGLER and DAY (1964). The test involved presenting the panel members with several samples along with a standard of water for reference. Each coded sample was compared individually to the reference to determine if there was an odour difference between them. Six samples were presented to each panelist during each session. The first bottle was a reference and contained only double-distilled water. The next five bottles consisted of the four different dilutions in order of increasing concentration and a water sample identical with the reference. Probably a better term for this method should be multiple paired comparison test. A group of 25 unscreened and untrained panelists (75% female and 25% mal ranging in age from 18 to 35 years with a mean of 26 years) was used for determining threshold values. In all cases, tests were repeated a sufficient number of times, so that a minimum of 50 responses were obtained for each test.

The samples of juice were evaluated for odour using a 5-point quality scale recommended for grapefruit juice quality control in Cuba (MINAL, 1979). In each test, 6 well-trained members of a sensory test panel evaluated the samples. The sum of scores produced for each sample was divided by the number of sensory test panel members and the quotient was used as the sensory score in this study.

1.4. Statistical analyses

The statistical analyses for determining the threshold values involved predicting the concentration that corresponded to 50% positive responses from the total judgements (PATTON & JOSEPHSON, 1957). The prediction was made from the regression of Y (percent of detection) on X (log of concentration in p.p.b.). The 95% confidence limit (LERCH, 1977) calculated for the threshold values was used as a measure of error. The relative flavour contribution was calculated as the ratio of the amound present in grapefruit juice and the odour threshold value of each compound.

The models for relating sensory scores (S) to gas-chromatographic data (C) are given in Table 1 where C is the component concentration (%) estimated

by GLC. These models have been suggested in analysis of other psychophysical contexts (PERSSON et al., 1973). The validity of the different models was tested by the F statistics and the correlation coefficient.

Table 1

Models for relating sensory scores with gas-chromatographic data

(I)	$S = a\,C + b$	linear form
(II)	$\log S = a \log C + b$	Stevens' law
(III)	$S = a \log C + b$	Fechner's law

S: sensory score.
C: concentration in p.p.b.

In addition to application of simple linear regression analyses, multiple linear regression analysis was used to improve the results. It was carried out on a CID 201 B computer (Cuba) using a stepwise program with F statistic as the criterion for deleting a variable.

2. Results and conclusions

Odour threshold concentrations were determined for aqueous solutions of hydrocarbons, aldehydes, ketones, alcohols and esters believed to contribute to grapefruit flavour. These threshold values are listed in Table 2 with the 95% confidence limits. Also listed in this table are the amounts normally present in grapefruit juice, where available (KIRCHNER et al., 1953; KIRCHNER & MILLER, 1953; PINO, 1984) and the relative flavour contribution of each volatile compound.

The procedure used for determining odour threshold is simple and the precision appears high according to the significant simple correlation coefficient and the narrow confidence limit of the compounds tested. Using this same procedure, AHMED and co-workers (1978) had demonstrated that odour and flavour threshold are identical.

The odour threshold values found in this study were generally in close agreement with those reported previously (GUADAGNI et al., 1963; BUTTERY et al., 1971; STAHL, 1973; AHMED et al., 1978) with a few exceptions. The reported odour threshold for octanal was 6 times lower (AHMED et al., 1978) and those for limonene, α-pinene and α-terpineol (AHMED et al., 1978) were 3, 7 and 70 times lower than those found in the present study. Disagreement in threshold values of some compounds in the literature and those obtained in this study could be due to differences in the purity of the compounds tested.

Of the five hydrocarbons evaluated, four contribute to grapefruit flavour. Limonene, which is by far the major component of grapefruit peel oil, is present

Table 2

Odour threshold value in water of selected volatile compounds present in grapefruit juice

Compound	Odour treshold (p.p.b.)			Conc. in grapefruit juice (p.p.b.)	Relative flavour contribution
	probable threshold	confidence limit (95%)	correlation coefficient		
α-pinene	62	42–82	0.97*	9 500[a]	153
sabinene	37	24–51	0.99**	1 000[a]	27
myrcene	46	45–47	0.99**	3 410[a]	74
limonene	229	212–245	0.96*	86 000[a]	376
p-cymene	6.2	1.5–10.9	0.97*		
acetaldehyde	8.7	0.6–16.8	0.99**	1 450[b]	167
butanal	8.2	0.1–16.3	0.89		
hexanal	6.9	1.2–12.6	0.89**		
octanal	6.4	5.2–7.5	0.96*	810[a]	127
nonanal	4.4	3.0–5.7	0.98*	140[a]	32
decanal	4.9	3.7–6.0	0.98*	490[a]	100
dodecanal	1.5	1.3–1.7	0.99**	200[a]	133
citral	28	10–45	0.92	220[a]	8
citronelal	31	18–44	0.98*	130[a]	4
furfural	282	261–303	0.93	0[a]	0
acetone	437 119	437 159–437 079	0.90	9 000[a]	0.02
carvone	6.7	0.1–13.3	0.98*	100[a,b]	15
nootkatone	800	750–850	0.98*	6 000[a]	8
methanol	369 828	369 863–369 793	0.93*	200[b]	0.001
ethanol	1.15×10^6	1.05×10^6–1.2×10^6	0.80	26 000[a]	0.02
3-methyl-1-butanol	1 005	986–1024	0.89	1 000[a]	1
2-hexanol	15.2	11.4–19.0	0.99*	1 000[a]	66
citronellol	10.6	0.1–21.1	0.95		
linalool	4.7	0.1–9.6	0.99**	160[a,b]	34
α-terpineol	4.6	3.1–6.1	0.78	30[a,b]	7
geraniol	4.1	3.3–4.8	0.99**	50[b]	12
epoxydihydro-linalool	1 148	1 141–1 155	0.99**	370[a,b]	0.3
ethyl acetate	8.5	7.5–9.5	0.98*	16 200[a]	1906
geranyl acetate	36	15–57	0.86	100[a]	3
neryl acetate	42	24–61	0.87	100[a]	2
methyl butyrate	15.1	14.7–15.5	0.99**	4 200[a]	278
ethyl butyrate	1.1	0.1–1.8	0.98*	10 000[a]	9091

* reported values are significant at 5% probability level.
** reported values are highly significant at 1% probability level.
[a] determined in the present study.
[b] KIRCHNER et al. (1953).

in juice at nearly 400 times its odour threshold level in water. The p-cymene has a much lower odour threshold than the other hydrocarbons but since its amount in grapefruit juice has not been determined, the degree of its contribution to grapefruit flavour is uncertain. BLAIR and co-workers (1952) demonstrated the presence of p-cymene in deteriorated orange juice due to the acid catalyzed hydration-dehydration of terpenes.

Carbonyl compounds play a major role in grapefruit flavour and the quality of its products is historically related to their aldehyde content and the nootkatone content (NAGY et al., 1977). The 10 aldehydes and 3 ketones in Table 2 were selected for study because they occur in relatively large amounts

in the juice or in the peel oil (NAGY et al., 1977; SHAW, 1979). Most of the carbonyl compounds in Table 2 are present in grapefruit juice in concentrations above threshold values, with the exception of furfural and acetone. For two of the aldehydes studied, the contents in grapefruit juice have not been reported.

Of the nine alcohols evaluated, five contribute greatly to grapefruit flavour. For three of them, the concentrations in grapefruit juice do not exceed the odour threshold as shown in Table 2. The concentration of citronellol has not been estimated in the juice. The most potent alcohols evaluated, 2-hexanol and linalool, have odour thresholds 66 and 34 times lower than their contents in grapefruit juice. The concentration of α-terpineol would be expected to increase by heat treatment and storage of the juice and it contributes to the off-flavour that develops in canned grapefruit juice during storage (KIRCHNER & MILLER, 1953).

Five esters were evaluated that may be important to grapefruit flavour. Ethyl acetate, methyl butyrate and ethyl butyrate probably are important contributors to the flavour of grapefruit juice since they all have fruity aromas.

As it was well known to us which volatile compounds would be essential to good sensory quality, we looked for causal relations between sensory response and GLC of such believed important constituents to grapefruit flavour. A typical GLC pattern of the volatiles isolated from juice is shown in Fig. 1. The identification of the major compounds was previously reported by PINO and co-workers (1985) as well as PINO (1984).

Using the models given in Table 1, simple linear regression analysis was carried out and the significant models are shown in Table 3. Since the odour tests were performed by an ordering method, a positive correlation in a peak means that it gives a preferable contribution, and a negative correlation means that this peak gives a reverse contribution with an increase in concentration. Of the 38 peaks used in the models, 10 give close correlations. The concentrations of nootkatone (peak No. 38), methyl butyrate (peak No. 5), ethyl butyrate (peak No. 7), limonene (peak No. 14) and decanal (peak No. 22) show a positive correlation with the sensory scores. Nootkatone has been considered a flavour-active compound in grapefruit products (MCLEOD & BUIGHES, 1964; STEVENS et al., 1970).

The concentrations of t-epoxydihydrolinalool (peak No. 20), c-epoxydihydrolinalool (peak No. 21) and α-terpineol (peak No. 27) give negative correlation with the sensory scores. These compounds have been reported as degradation products in aged grapefruit juice (KIRCHNER & MILLER, 1953). The other two negatively contributing peaks are No. 25 and No. 34, which could not be identified.

Judging from the results, there are good reasons to find models for relationships between sensory response and GLC. Determining the odour threshold values and the concentrations of 28 constituents, we found 23 constituents

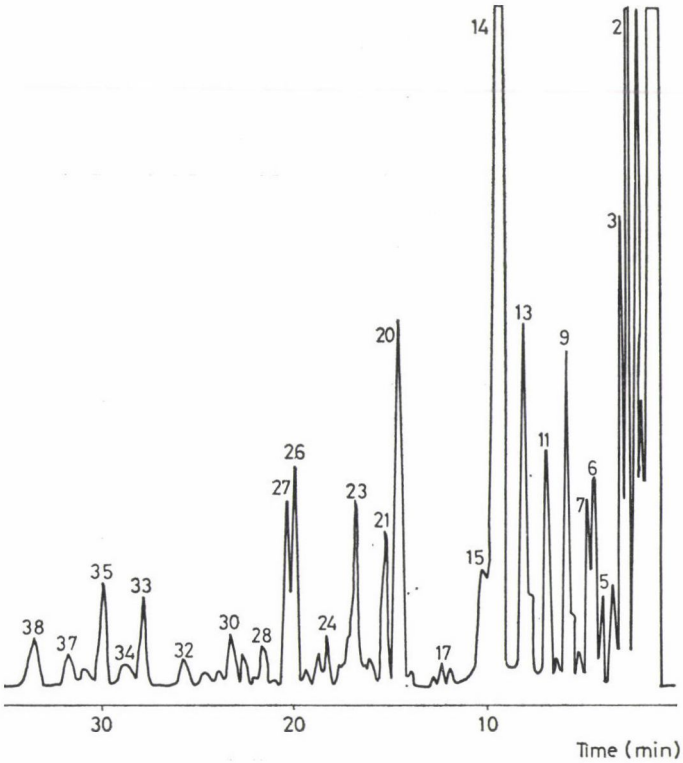

Fig. 1. Separation by GLC of the volatile constituents from grapefruit juice on Carbowax 20 M column. Experimental conditions are given in Materials and methods. The peaks are identified in Table 3

which appeared to be important to grapefruit flavour, that is, they are close or above threshold values. But only 8 of them are in close correlation with the sensory scores. Well known to flavourists, perfumers and others who work with sensory materials, are the facts that certain compounds mask other compounds or exhibit additive, synergistic or antagonistic effects when they are in combination, that a compound unpleasant by itself may be essential to good sensory quality, and that the concentration of a compound often affects pleasantness and if it changes could lead to unpleasantness. Some of these facts must explain why nootkatone which is only 8 times above its threshold value is more important than limonene which is nearly 400 times above its threshold value. For these reasons, correlation analysis was needed to provide some idea of which volatile compounds are important to grapefruit flavour.

The data of the experiments were subjected to multiple linear regression analysis which concluded at the last step with peak No. 38 alone and significant at 0.1% probability level, so nootkatone is the most important constituent responsible for the flavour quality of grapefruit juice.

Table 3

Simple linear regression models of correlations between sensory response and GLC of grapefruit juice volatiles[a]

Peak No.	Constituent	Correlation	$R^2 \times 100$
	Linear form		
20	t-epoxydihydrolinalool	—	35.4
21	c-epoxydihydrolinalool	—	27.3
25	unknown	—	32.1
27	α-terpineol	—	58.1
34	unknown	—	27.8
38	nootkatone	+	85.6
	Stevens' law		
5	methyl butyrate	+	49.0
7	ethyl butyrate	+	61.3
14	limonene	+	40.9
20	t-epoxydihydrolinalool	—	31.6
22	decanal	+	48.2
27	α-terpineol	—	43.2
34	unknown	—	38.1
38	nootkatone	+	81.7
	Fechner's law		
5	methyl butyrate	+	52.6
20	t-epoxydihydrolinalool	—	33.9
22	decanal	+	40.9
25	unknown	—	30.0
27	α-terpineol	—	46.5
34	unknown	—	39.8
38	nootkatone	+	86.9

[a] reported values are highly significant at 1% probability level.

As a measure of how well the calculated sensory score agrees with the observed sensory score, the correlation coefficient and the regression line according to the two sets of data were calculated for the linear model with nootkatone. The results are shown in Fig. 2. Ideally, the numerical value of the slope should be equal to unit and the intercept be zero, but the differences did not differ significantly at the 5% probability level and therefore they are of no practical importance. Thus, the calculated sensory scores using the GLC are as representative as the sensory scores determined by the sensory test panel.

Summing up, it can be said that in this study a definitive contribution to grapefruit flavour was indicated for certain volatile constituents of grapefruit juice. Thus, methyl butyrate, ethyl butyrate, decanal, limonene and particularly nootkatone, contribute significantly to the pleasantness of grapefruit flavour. A few constituents of grapefruit juice, t-epoxydihydrolinalool, c-epoxydihydrolinalool and α-terpineol contribute to unpleasantness of flavour when their concentrations increase during storage of the juice. Certain other constituents of grapefruit are present at or slightly above their flavour threshold levels in the juice and probably also contribute to grapefruit juice flavour,

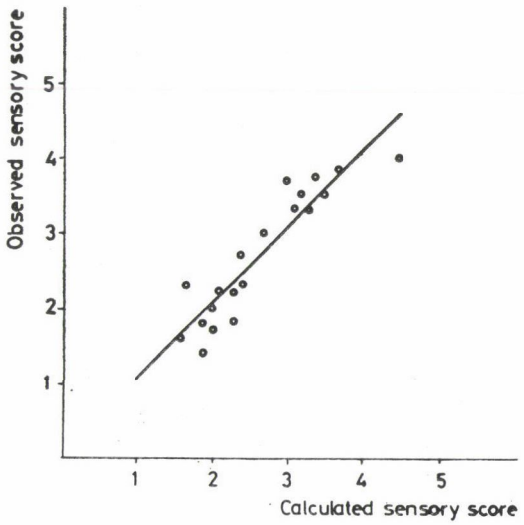

Fig. 2. Comparison of the observed sensory score (S_o) and the one calculated (S_c) by the linear model with nootkatone. ($S_o = 0.02 + 1.01\ S_c$; $r = 0.9$)

while others are present in quantities below their threshold levels and the concentrations for several others have not been determined.

The regression models using the nootkatone concentration determined by GLC could be used to describe the sensory quality of a grapefruit juice.

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BOOK REVIEWS

Amino acid composition and biological value of cereal proteins

R. LÁSZTITY and M. HIDVÉGI (Eds.)

Proceedings of the International Association for Cereal Chemistry Symposium

Akadémiai Kiadó, Budapest, 1985; 662 pages

This book contains the edited proceedings of the International Symposium of the International Association for Cereal Chemistry held in Budapest, 1983. In addition to the lectures presented on the Symposium the book also contains articles of invited contributions. In this way the book written on 662 pages and published by the Akadémiai Kiadó, Budapest, can be regarded as the most modern handbook in this field of science. It contains a number of theoretical and practical procedures and methods, numerous measuring results and also gives the amino acid compositions of various materials formulated in tables. The calculation methods of the biological value of cereal proteins are also included which is very essential. The subject index, given on more than 50 pages, makes the book a valuable source of information to a great extent. An unique value of the volume for the specialists in this field is that all the names, professions, post addresses of the authors can be found in it offering great possibilities for direct international exchange of experiences.

L. SZALA

Analysis of food carbohydrates

G. G. BIRCH (Ed.)

Elsevier Applied Science Publishers Ltd., Barking, Essex, England, 1985; 311 pages

It was the aim of the book to provide a complete collection of the methods of carbohydrate analysis which is useful not only for scientists and technical experts working in food research and industry but for those interested in control of food quality, in nutrition, biology or agriculture, too.

As introduction the general properties of food carbohydrates, their structure, their digestibility, absorption and other physiological characteristics are described. The correlation between the sense or intensity of taste, on one hand, and the molecular structure on the other, are shown. An account is given of the possibilities of determining relative sweetness.

First the simpler and quicker physical methods of carbohydrate determination are discussed, such as polarimetry, refractometry and hydrometry. Subsequently the chromatographic methods are described, including their theoretical principles and technical process.

Of the methods detecting sugars by their reducing capacity the Fehling method, Munson and Walker method, and the Luff-Schoorl method are mentioned, of the colorimetric methods Nelson-Somogyi's method, the 2,3,5-triphenyl tetrazolium chloride method and the method utilizing 3,5-dinitrosalicylic acid reagent, etc.

Of the chromatographic methods HPLC (high performance liquid chromatography) is discussed in detail, including sample preparation, clarifying and other problems related to detection. In relation to GLC (gas liquid chromatography) the preparation of the carbohydrate derivatives, of the necessary reagents and other problems are discussed.

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In following up syntheses various chromatographic techniques may be helpful. The usability of thin layer chromatography (TLC), gas liquid chromatography (GLC), HPLC, infrared spectroscopy (IRS), nuclear magnetic resonance spectroscopy (NMR) and polarimetry, is discussed. Separation of the products and various modern techniques to detect structure, are treated.

The utilization in structure research of nuclear resonance spectroscopy is treated in detail.

In the chapter on food glycoside analysis the structure, formation and significance of the most important glycosides (glycoalkaloid, cyanogen glycosides, phenolic glycosides, thioglycosides, saponiens), are given. The two main types of analysis, the main steps of procedure and methods serving to detect the structure of the aglycon part and the sugar residue formed upon hydrolysis, are discussed.

In the last chapter the metabolism in the digestive tract, the absorption of simple sugars, the hydrolysis of strach, the polysaccharides soluble in water and the fibres, are described. Beside the chemical processes enzymic methods are also investigated.

The book is provided with a great number of tables, spectra, an ample list of literary references and it is considered to achieve its purpose.

Mrs J. Bogdán

Food industries and the environment

(Proceedings of the International Symposium Budapest, Hungary, 9—11 September, 1982)

J. Holló (Ed.)

Akadémiai Kiadó, Budapest, 1984; 565 pages

Two vital topics of our age meet on the pages of this collection of essays: food production and protection of environment. It is no more change that these problems formed the subject of a symposium held in Hungary, because the increase of food production in Hungary requires development in both spheres.

The volume containing 63 essays presents on 565 pages the subjects of papers in 54 publications in English and 9 publications in French. Distribution according to subject of the papers:

General problems are treated in 17 papers, problems of the canning industry in 7, of the dairy industry in 8, of the meat industry in 18, of the syrup, starch, sugar and alcohol industries in 7 papers, while 6 papers discuss problems related to noise, vibration, dust and odour, detrimental to human health.

The authors of 39 papers were foreign experts and 24 Hungarian ones. In accordance with the closing address of Mr Guy Dardenne, the present situation can be best assessed by the changes occurring in the relationship between food manufacture and agricultural production which started with the upsetting of equilibrium around the 960s and lasts up to the present.

While in the stage of this so called equilibrium it was aimed at the utilization of by-products (in the sugar industry it was tried to return to agriculture the cossettes and the beet sludge and in the distilling industry the mash) and fresh waters were only slightly loaded, and so was the environment, in the next period the concentration of production, changes in the price and value relations brought about the breaking down of this equilibrium, built on compromises. This latter period caused the look-out for new ways in the utilization of by-products, the best possible utilization of the bio-mass in order to protect the human environment.

At the beginning of this period the protection of the environment was subject to legal regulation and those who violated the rules were fined to in increasing extent. However, it was established that this is not a satisfactory, effectual way of preventing pollution of the environment. To build up an organization to keep pollution under control would have involved high expenses. Thus, nowadays the tendency is to select production technologies permitting protection of the environment and the utilization of possible by-products is planned. The latter aims are widely discussed in the papers and directives are provided to solve pollution problems.

Simultaneous, complementary utilization of agricultural produce as food or feed or industrial raw material or source of energy requires adequate organization.

The volume of essays shows that procedures and technologies successful in other fields are borrowed by the food industries. Lyophilization was taken over from the pharmaceutical industry, certain methods of protein processing from the textile industry and the chemical industry.

Thus, in order to protect the environment it is essential to utilize every possible means and procedures. Environmental sanitation is a increasing requirement of society and this has to be met.

The responsibility of experts and politicians is on the increase, therefore they expect representatives of different disciplines to find new ways and solutions to the problems of environmental protection, as it is indicated in the introduction by Professor Holló.

The essays in this collection are foreshadowing progress in this field and therefore they are theoretical as well as of practical value to experts in the food industry.

P. STEFANOVITS

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IDENTIFICATION OF VOLATILE FLAVOUR COMPONENTS OF ROASTED WHITE SESAME SEED

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Volatile flavour components of roasted sesame seeds were studied. The volatile components were obtained by roasting under reduced pressure. The volatile extract was separated into neutral-acidic and basic fractions. The neutral--acidic fraction was further separated into carbonyl and non-carbonyl components. All fractions were analysed by gas liquid chromatography and mass spectrometry. Thirty-two constituents, including 10 aldehydes, 5 ketones, 6 alcohols, 4 esters and 7 pyrazines were identified. Among them 4-(5-methyl-2-furyl)-3-buten-2-one is claimed to be reported for the first time in the aroma of roasted white sesame seeds.

Keywords: white sesame seeds; aroma: neutral-acidic, basic; 4-(5-methyl-2-furyl)-3-buten-2-one.

Sesame is an important oil-seed and has been extensively cultivated in tropical and subtropical areas since ancient times. Egypt is one of the main sesame seed producing countries. The local varieties of sesame seeds are utilised in a large number of candy and baked products in addition to oil production.

Extensive studies have been carried out concerning the nutritional value and chemical composition of sesame seeds but few have focused on flavour components of the roasted seeds, especially the local varieties, which are usually roasted before crushed or admixed.

SOLIMAN and co-workers (1974) identified *n*-octanol, benzyl alcohol and 2-furfuryl alcohol in the aroma of roasted sesame seeds, claiming to be the source of the aroma of roasted sesame seeds. MANLEY and co-workers (1974) also investigated the basic compounds from roasted sesame seeds and reported them to be mainly alkyl pyrazine derivatives.

The use of modern analytical techniques, particularly gas chromatography and mass spectrometry have revealed the complexity of the aroma in heat-processed foodstuffs. These numerous studies of complex flavour component systems were reported in the aroma of roasted materials such as filberts (KINLIN et al., 1972), almond (TAKEI et al., 1974), cocoa (VITZTHUM et al.,

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1975), cooked rice (YASHIMA et al., 1978) and pistachio nut (SOLIMAN et al., 1981).

The present work deals with the identification of the volatile components of the aroma of roasted white sesame seeds, in addition to the study of the role of lipid constituents, carbohydrates and amino acids in producing this aroma.

It is a normal procedure that for the preparation of aroma concentrate from any source, the material used is to be divided into two parts, every part is subjected to the same treatment for collecting the aroma concentrate. Then analyses of the products are carried out separately under the same conditions for comparison. The data obtained in every case are always repeatable within the ordinary limits of experimental errors.

1. Materials and methods

1.1. Materials

The local sesame seeds *(Sesamum indicum)* variety Giza 23 (white) was kindly supplied by the Department of Horticulture, Ministry of Agriculture (Egypt). Most of the authentic reference compounds used were kindly supplied by the International Flavours and Fragrances, Inc. (New Jersey, U.S.A.) and Ochanomizu University, Food Chemistry Laboratory (Japan). All other reagents and solvents were extra pure or analytical grade.

1.2. Lipid extraction

Clean crushed white sesame seeds (3.500 kg) were subjected to *n*-hexane extraction using Soxhlet's extraction apparatus to isolate the lipid which amounted to 57% of whole seeds.

1.3. Preparation of the aroma concentrate

1.3.1. Roasted whole seeds. Clean crushed white sesame seeds (0.600 kg) were roasted using silicon oil bath at 453 K for about two hours under reduced pressure (about 2000 Pa) with a continuously rotating device, yielding 0.00424 kg of aroma concentrate (SOLIMAN et al., 1974).

1.3.2. Roasted, lipid-free seeds. Lipid free crushed seeds (0.800 kg) were used for the preparation of aroma concentrate by the same method mentioned above, yielding 0.00487 kg of aroma concentrate.

1.3.3. Heated sesame lipids. Sesame lipids (0.250 kg) were treated in the same way yielding 0.0012 kg of aroma concentrate.

1.4. Preparation of aroma concentrate fractions

Fractionation of the aroma concentrate was carried out as follows:

The aroma concentrate was fractionated into neutral-acidic and basic fractions according to TAKEI and co-workers (1974). This process was applied to the aroma concentrate from:

— Roasted whole white sesame seeds; 0.00423 kg of the aroma concentrate yielded 0.00259 kg neutral-acidic and 0.00164 kg basic fractions.

— Roasted lipid-free seeds; 0.00487 kg of the concentrate gave 0.0035 kg neutral-acidic and 0.00128 kg basic fractions.

The neutral-acidic fraction of the whole white sesame seeds (0.00259 kg) was further fractionated into carbonyls (0.001079 kg) and non-carbonyls (0.00152 kg) using Girard-T-reagent by the method of TEITELBAUM (1958).

1.5. Gas-liquid-chromatographic analysis

This was done using a dual flame ionization detector Pye Unicam instrument series 104 (England). The column was packed with 20% diethylene glycol succinate (DEGS) Chromosorb W (60–80 mesh); carrier gas flow rate 0.00075 $dm^3\ sec^{-1}$ (He); column temperature 343–468 K with programming rate 277 K min^{-1}. Injection port temperature was 473 K. The chart speed was set at 0.013 cm sec^{-1}.

1.6. Gas-liquid chromatography–mass spectrometry (GLC-MS) coupling

The identification of the components was achieved using GLC-MS coupling apparatus, Varian 1400-Mat 112 (Varian, Zug, Switzerland) under the following conditions: column: 3% SE 30 on Chromosorb W 100–120 mesh, 1.9 m length 0.003 m internal diameter used in identification and confirmation of the structure. Column temperature: 343–463 K, programming rate: 277 K min^{-1}, chart speed: 0.013 cm sec^{-1}. Flow rate (He): 0.00033 $dm^3\ sec^{-1}$, temperature of ion source of mass spectrometry: 473 K under reduced pressure of 133.23×10^{-6} Pa and the electron voltage was 70 eV.

2. Results and discussion

2.1. Aroma of whole white roasted sesame seeds

The gas chromatograms of aroma concentrate (*a*) as well as its neutral-acidic (*b*) and basic (*c*) fractions are shown in Fig. 1, and those of the corresponding carbonyl (*d*) and non-carbonyl (*e*) fractions are shown in Fig. 2. The components together with their concentrations as obtained from GLC analysis are reported in Table 1.

2.2. Neutral-acidic fraction

The neutral-acidic fraction (*b*) has roasted sesame seed aroma with oily flavour. Some aliphatic aldehydes, heterocyclic ketons and other compounds were identified.

2.2.1. Carbonyl fraction (d). This fraction has sweet oily flavour. Among twenty-two compounds, only fifteen could be identified. From Table 1 it can

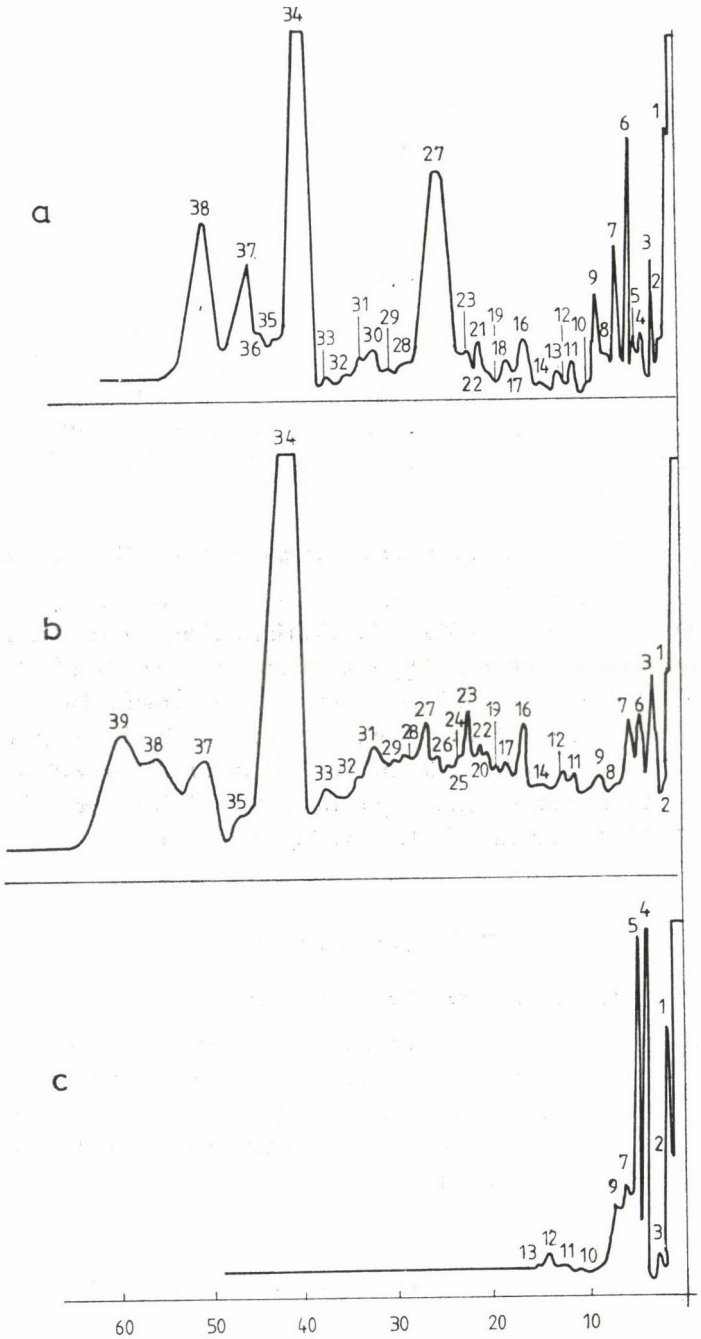

Fig. 1. Gas-liquid chromatograms of the aroma concentrate of roasted whole white sesame seeds and its neutral-acidic and basic fractions, respectively

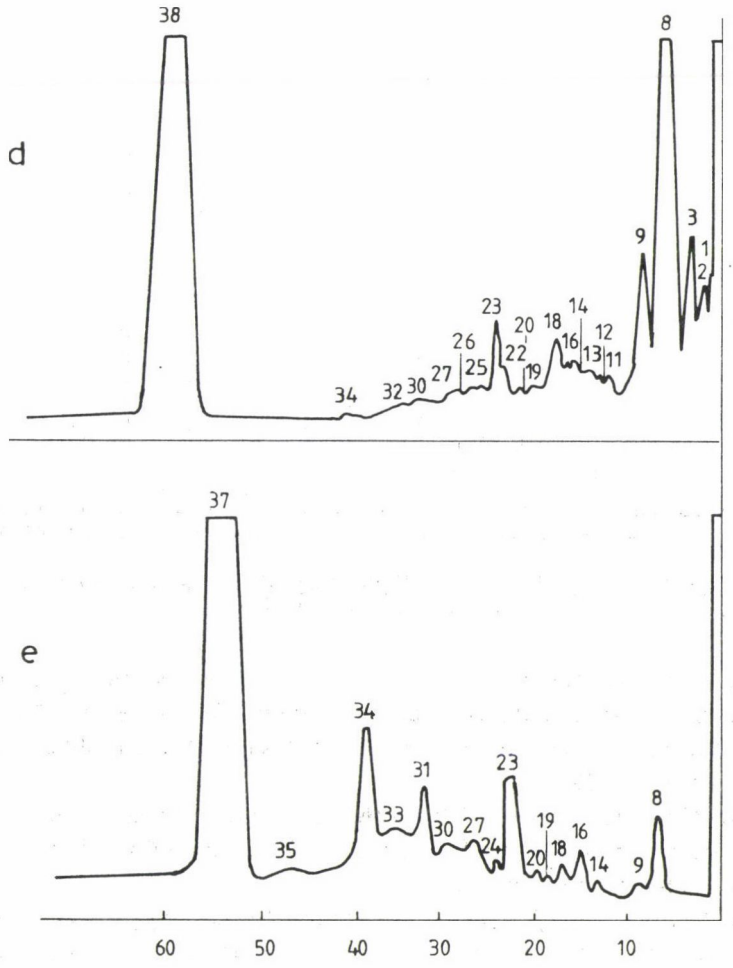

Fig. 2. Gas-liquid chromatograms of carbonyl and non-carbonyl fractions, respectively

be seen that octanal and 2,4,6-dodecatrienal are the predominant constituents, as represented by peak area percentage 30.0 and 10.55, respectively. These compounds are believed to be liberated from the lipid fraction (SCHULTZ et al., 1962) and might be responsible for the oily flavour of the carbonyl fraction.

Furan derivatives are claimed to be responsible for the sweet pleasant aroma of the carbonyl fraction.

However, it is suggested that 3-methylbutanal, benzaldehyde, 2-furfural, 5-methylfurfural, 2-acetylfuran, 4-(5-methyl-2-furyl)-3-buten-2-one were developed by caramelization of any sugar moiety over 463 K (BRYCE & GREENWOOD, 1963; TAKEI et al., 1969; SOLIMAN et al., 1981). Except 4-(5-methyl-2-

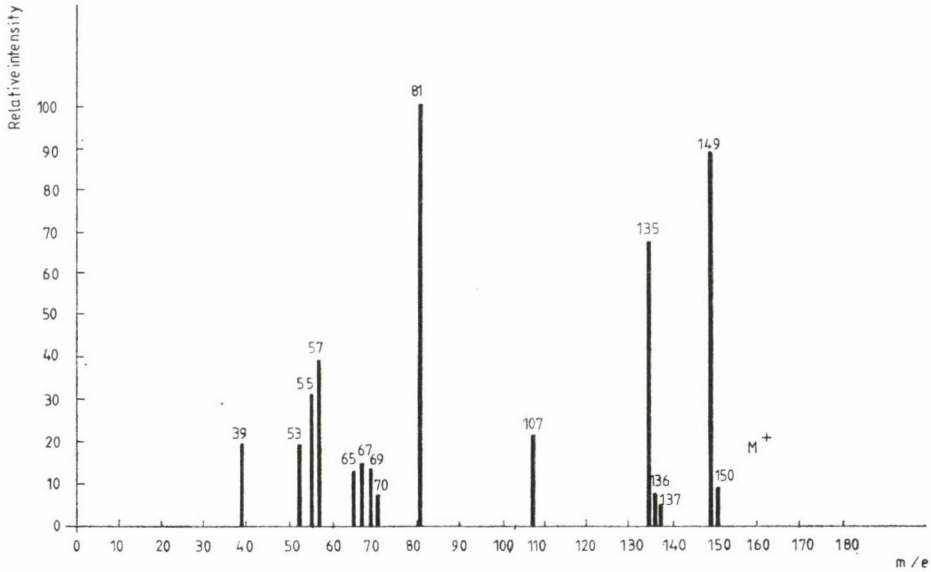

Fig. 3. Mass spectrum of peak 27/4-(5-methyl-2-furyl)-3-buten-2-one. m/e: Mass-to-charge ratio; M^+: molecular ion

furyl)-3-buten-2-one, the aforementioned compounds were reported in the aroma of some roasted materials such as filbert (KINLIN et al., 1972), white Japanese sesame seeds (SOLIMAN et al., 1974; TAKEI et al., 1969) and pistachio nut (SOLIMAN et al., 1982).

Thus, the presence of 4-(5-methyl-2-furyl)-3-buten-2-one (Fig. 3) is claimed to be reported for the first time in the aroma of whole roasted white sesame seeds under investigation.

2.2.2. Non-carbonyl fraction (e). This fraction has typical roasted sesame seed flavour. Among the sixteen compounds separated, nine were identified. Table 1 indicates that methylundecanoate is the major component (53.93%), although it has no distinct flavour. The compounds *n*-octanol, benzyl alcohol and 2-furfuryl alcohol are represented by concentrations of 3.19, 6.20 and 1.76%, respectively, and might be considered as criteria of the aroma of roasted sesame seeds (SOLIMAN et al., 1974).

2.3. Basic fraction

The components of the basic fraction (*c*) with its characteristic roasted nut-like aroma were identified, either by the retention time of authentic sample or by the mass spectrum data. Seven pyrazine derivatives were identified. However, similar findings were observed in the volatile compounds of roasted filberts (KINLIN et al., 1972), roasted sesame seeds (MANLEY et al., 1974), peanut (WALRADT et al., 1971) and almond (TAKEI et al., 1974).

Table 1

The composition of aroma concentrate from roasted white sesame seeds and its neutral-acidic and basic fractions
(Reference to Figs. 1, 2)

Peak No.	t_R (min)	$t_{R,F}$	Concentration (%)					Compounds	Method of identification	
			Aroma concentrate	Neutral-acidic fraction	Carbonyl fraction	Non-carbonyl fraction	Basic fraction			
1	1	0.6	2.79	9.0	1.57	—	14.64	2-Methylpyrazine, 3-ethanal	M.S.	t_R
2	2.3	0.13	0.43	1.02	4.34	—	23.96	2-Ethylpyrazine, 3-methylbutanal		t_R
3	2.5	0.15	1.51	2.79	6.31	—	3.00	Propylpyrazine, hexanal		t_R
4	4.5	0.27	1.07	—	—	—	17.99	2,3-Dimethylpyrazine		t_R
5	5.0	0.30	0.49	—	—	—	27.14	2,5-Dimethylpyrazine	M.S.	
6	6.0	0.36	4.81	1.56	30.00	—	—	Octanal		t_R
7	6.5	0.39	2.70	1.64	6.31	—	2.55	2,3-Diethylpyrazine, nonanal		t_R
8	8.0	0.48	0.21	0.11	—	3.19	6.25	2,5-Diethylpyrazine, n-octanol		t_R
9	8.5	0.51	1.81	0.66	—	0.67	—	Nonyl alcohol		
10	10.0	0.60	0.19	—	—	—	0.56			
11	11.0	0.65	0.76	0.40	0.74	—	1.99	Benzaldehyde, unknown		t_R
12	10.5	0.74	0.29	0.27	0.37	—	1.71			
13	13.0	0.77	0.29	—	0.21	—	0.21			
14	14.2	0.84	0.06	0.05	0.32	0.73	—			
15	16.2	0.96	0.38	—	0.74	—	—			
16	16.8	1.00[b]	0.49	1.00	1.98	2.20	—	2-Furfural		t_R
17	18.0	1.07	Trace	0.05	—	—	—			
18	18.5	1.10	0.76	0.17	—	1.99	—			
19	20.0	1.19	0.08	0.12	0.40	0.73	—	5-Methylfurfural, undecanol	M.S.	t_R
20	20.5	1.22	0.05	0.18	0.56	1.09	—	2-Acetylfuran, dodecanol	M.S.	t_R
21	22.0	1.31	0.41	0.20	—	—	—			
22	22.5	1.34	0.05	0.05	1.37	—	—	Heptyl ester (Me-heptanoate)	M.S.	t_R
23	23.2	1.39	0.33	0.36	2.3	5.02	—			
24	24.0	1.43	—	0.06	—	0.33	—			
25	14.5	1.48	—	0.04	0.21	—	—			
26	26.5	1.67	—	0.06	0.45	—	—	3-Heptanone	M.S.	
27	28.0	1.67	17.88	0.59	0.51	2.95	—	4-(5-methyl-2-furyl)-3-butene-2-one	M.S.	
28	30.0	1.79	0.23	0.08	—	—	—	Octyl ester (Me-hexanoate)	M.S.	t_R
29	30.5	1.82	0.11	0.07	—	—	—	3-Octanone	M.S.	
30	32.5	1.93	1.25	—	0.20	2.99	—	Nonyl ester (Me-hexanoate)	M.S.	
31	34.5	2.03	0.36	0.9	—	6.20	—	Benzyl alcohol		t_R
32	35.0	2.08	0.06	1.08	Trace	—	—	3-Nonanone		
33	38.3	2.29	0.39	1.44	—	1.76	—	2-Furfuryl alcohol		t_R
34	40.0	2.50	36.60	49.44	Trace	12.83	—	2,4-Undecadienal	M.S.	t_R
35	48.0	2.26	0.27	1.45	—	3.39	—			
36	41.0	3.04	0.54	—	—	—	—			
37	53.0	3.15	5.57	8.2	—	53.93	—	Methylundecanoate	M.S.	
38	58.0	3.45	16.82	6.90	10.55	—	—	2,4,6-Dodekatrienal	M.S.	
39	61.0	3.63	—	9.86	—	—	—			

Concentration relative to peak area (w %); t_R: retention time, $t_{R,F}$: retention time relative to 2-furfural; the number of measurements (n) = 3

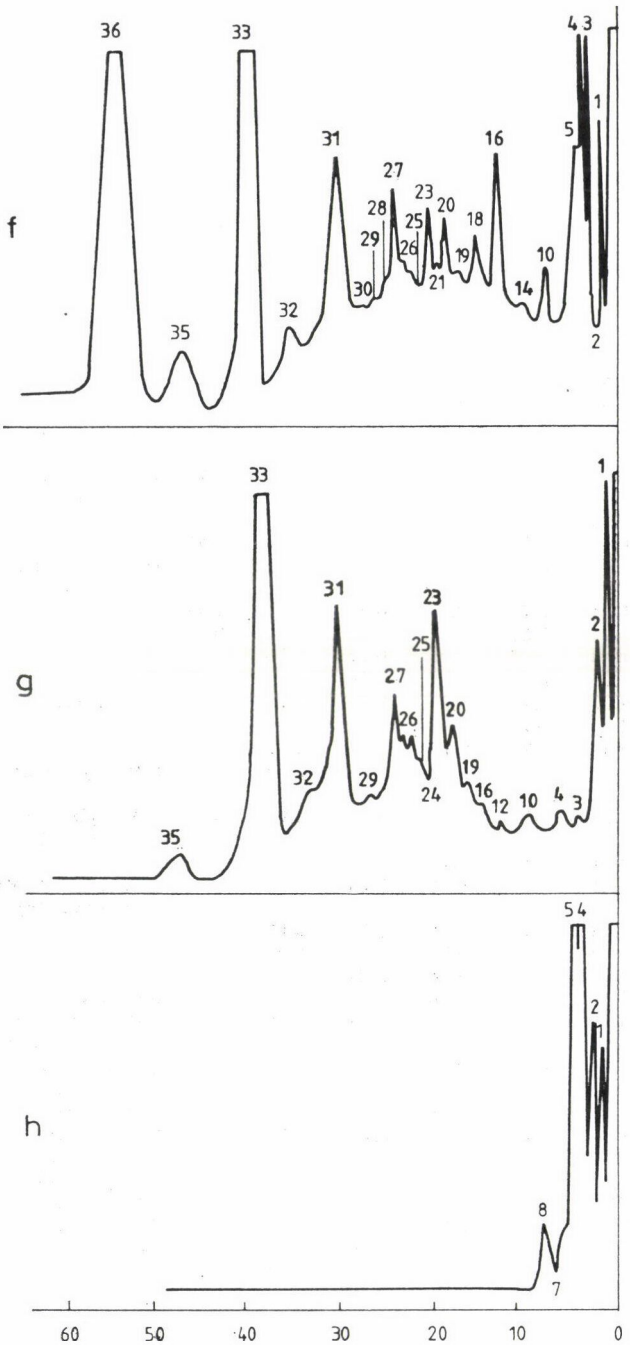

Fig. 4. Gas-liquid chromatograms of the aroma concentrate of roasted defatted white sesame seeds and its neutral-acidic and basic fractions, respectively

The data cited in Table 1 revealed that some pyrazine derivatives, namely, 2-methylpyrazine (14.64%), 2-ethylpyrazine (23.96%), 2,3-dimethylpyrazine (17.99%), 2,5-dimethylpyrazine (27.14%) are major components, while propylpyrazine, 2,3-diethylpyrazine and 2,5-diethylpyrazine are present in relatively low concentrations of 3.00, 2.55 and 6.25%, respectively.

2.4. Aroma of lipid-free white sesame seeds

The gas chromatograms of aroma concentrate (*f*) as well as its neutral-acidic and basic fractions (*g*, *h*) are shown in Fig. 4, while the corresponding concentrations are reported in Table 2.

2.5. Neutral-acidic fraction

The neutral-acidic fraction *(g)* has typical roasted sesame seed aroma. Its components were identified by comparison of their retention time with those of identified compounds of the aroma of whole white roasted sesame seeds. Among twenty compounds, ten were identified. It is obvious that the compounds present in the neutral-acidic fraction of defatted seeds were almost similar to those of whole seeds except octanal, *n*-octanol, nonanol, undecanol, dodecanol; 2,4-undecadienal, methyl undecanoate and 2,4,6-dedecatrienal which might be of lipid origin.

It is noteworthy that 2-furfuryl alcohol is the single predominating compound levelling up to 72.66%. This was in favour of the previous findings by SOLIMAN and co-workers (1974). The newly reported compound, 4-(5-ethyl-2-furyl)-3-buten-2-one, is represented by a much higher concentration (2.55%) compared to that of whole roasted seeds (0.59%). The other furan derivatives, though reported to be present at low concentrations, yet, greatly affecting the aroma giving rise to a typical strong sweet roasted seed flavour.

2.6. Basic fraction

Six pyrazine derivatives were identified in this fraction (*h*). The comparison of these derivatives in both the defatted and whole seeds, revealed that 2,3-dimethyl and 2,5-diethylpyrazine showed approximately the same concentration while the other pyrazine derivatives revealed considerable variation.

2.7. Aroma of heated lipids

It should be emphasized that the aroma concentrate obtained from sesame lipids *(i)* consists essentially of the same type of compounds identified before in case of roasted whole white sesame seeds. Some of these flavour

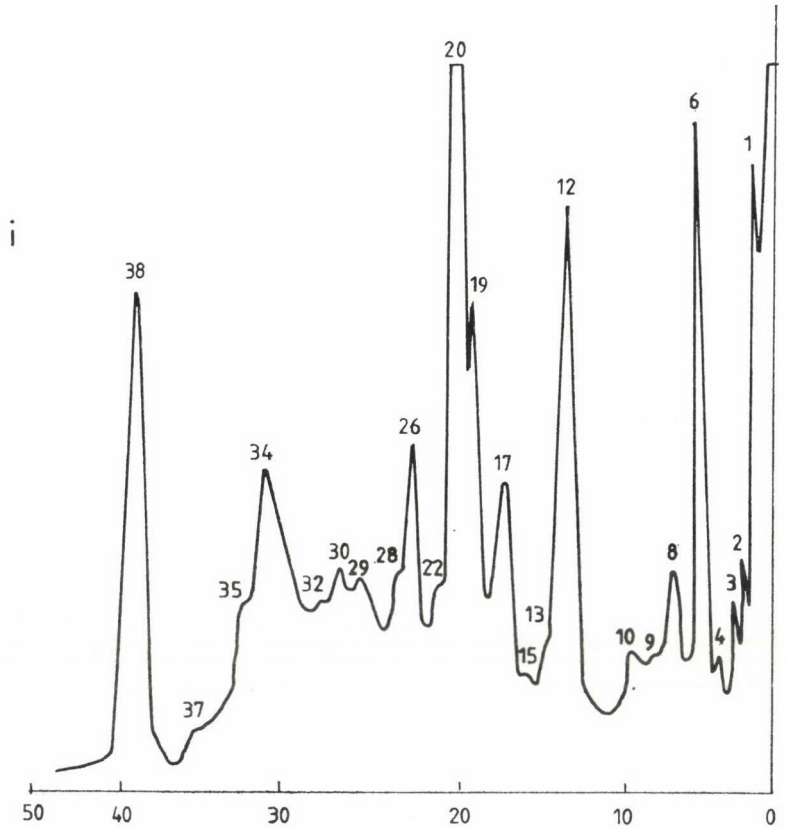

Fig. 5. Gas-liquid chromatograms of the aroma concentrate of heated oil

compounds were detected in higher concentration, for example, aldehydes (ethanal, 3-methylbutanal, hexanal, octanal), alcohols (octanol, undecanol, dodecanol), esters (methylhexanoate, methylheptanoate) and ketones (3-hexanone, 3-octanone, 3-nonanone, 3-decanone), in contrast to small amounts obtained for whole roasted seeds (Fig. 5 and Table 3). However, it is reported that these compounds might be derived from the lipids (GEHRIG & KNIGHT, 1961).

3. Conclusions

An overall survey of the above mentioned results, revealed that besides the variations of the identities and quantities of the components within the different samples, some general conclusions may be drawn as follows:

The neutral-acidic fraction is mainly responsible for the roasted sesame seed aroma. Of the two subfractions, the non-carbonyl fraction has typical roasted sesame seed flavour while the carbonyl fraction sweet oily flavour.

Table 2

The composition of aroma concentrate from roasted defatted white sesame seeds and its neutral-acidic and basic fractions
(Reference to Fig. 4)

Peak No.	Concentration (%)			Compounds
	Aroma concentrate	Neutral-acidic fraction	Basic fraction	
1	3.70	4.00	23.14	2-Methylpyrazine, ethanal
2	0.17	1.45	10.03	2-Ethylpyrazine, 3-methylbutanal
3	4.82	0.20	—	Hexanal
4	5.06	0.25	20.66	2,3-Dimethylpyrazine, unknown
5	0.75	—	33.50	2,5-Dimethylpyrazine
6	—	—	—	
7	—	—	5.63	2,3-Diethylpyrazine
8	—	—	7.04	2,5-Diethylpyrazine
9	—	—	—	
10	1.32	0.93	—	
11	0.54	Trace	—	Benzaldehyde
12	3.67	0.25	—	
13	—	—	—	
14	—	—	—	
15	—	—	—	
16	1.48	0.60	—	2-Furfural
17	—	—	—	
18	—	—	—	
19	0.24	0.42	—	5-Methylfurfural
20	0.78	1.17	—	2-Acetylfuran
21	—	—	—	
22	0.27	—	—	
23	1.55	4.10	—	
24	—	0.50	—	
25	0.30	0.40	—	
26	0.08	0.35	—	
27	1.25	2.55	—	4-(5-methyl-2-furyl)-3-butene-2-one
28	0.49	—	—	
29	0.16	0.46	—	
30	Trace	—	—	
31	5.20	4.10	—	Benzyl alcohol
32	3.05	1.53	—	
33	27.20	72.66	—	2-Furfuryl alcohol
34	—	—	—	
35	4.03	4.08	—	
36	33.53	—	—	

The alkaline fraction has roasted nut-like aroma.

For the defatted seeds, the neutral-acidic fraction has typical roasted sesame seed aroma: this is due to the disappearance of most of the components originating from the lipid fraction and to the higher concentration of the furan derivatives; the latter are responsible for the sesame seed like aroma, especially 2-furfuryl alcohol.

The lipid fraction of sesame seed is responsible for the oily flavour without playing significant role in the development of the characteristic aroma.

The 4-(5-methyl-2-furyl)-3-buten-2-one is claimed to be reported for the first time in the aroma of whole roasted seeds.

Table 3

The composition of aroma concentrate from roasted white sesame seeds and its heated oil
(Reference to Fig. 5)

Peak No.	Concentration (%)		Compounds
	Aroma concentrate of white sesame seeds	Aroma concentrate of heated oil	
1	2.79	4.87	2-Methylpyrazine, ethanal
2	0.43	3.42	2-Ethylpyrazine, 3-methylbutanal
3	1.51	4.26	Hexanal
4	1.07	1.53	2,3-Dimethylpyrazine
5	0.49	—	2,5-Dimethylpyrazine
6	4.81	22.24	Octanal
7	2.70	—	2,3-Diethylpyrazine, nonenal
8	0.21	2.23	2,5-Diethylpyrazine, n-octanol
9	1.81	0.36	Nonyl alcohol
10	0.19	3.1	
11	0.74	—	Benzaldehyde
12	0.29	11.05	
13	0.29	0.08	
14	0.06	—	
15	0.38	0.13	
16	0.49	—	2-Furfural
17	Trace	4.69	
18	0.76	Trace	
19	0.08	3.39	5-Methylfurfural, undecanol
20	0.05	16.13	2-Acetylfuran, dodecanol
21	0.41	—	
22	0.05	0.44	Heptyl ester (Me-hexanoate)
23	0.33	—	
24	—	—	
25	—	—	
26	—	3.54	3-Heptanone
27	17.88	—	4-(5-methyl-2-furyl)-3-butene-2-one
28	0.23	0.51	Octyl ester (Me-heptanoate)
29	0.11	0.53	3-Octanone
30	1.25	0.36	Nonyl ester (Me-octanoate)
31	0.34	—	Benzyl alcohol
32	0.06	0.23	3-Nonanone
33	0.39	0.88	2-Furfuryl alcohol, decanone
34	36.6	3.36	2,4-Undecadienal
35	0.87	1.93	
36	0.51	—	
37	8.87	0.77	Methylundecanoate
38	16.88	10.00	2,4,6-Dodecatrienal

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FERMENTED VEGETABLE COCKTAILS

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Cocktails prepared from enzymatically solubilized vegetables, represent a high nutritional value, containing almost all the vitamins and nutrients of the original tissue. Preservation of these valuable constituents of the cocktails was attempted by natural means, namely by lactic acid fermentation. Enzyme treatment and lactic acid fermentation of the sliced vegetables were carried out simultaneously at 30 °C for 22 hours on a shaker. *Lactobacillus plantarum*, *Lactobacillus helveticus*, *Streptococcus lactis* and starter cultures of yogurt and kefir manufacture were used for the fermentations. Proliferation and lactic acid production of the cultures was the highest at pH values of 4.5 and 5.6, while the sensory properties of the fermented samples proved to be better at lower pH values (pH 3.6–3.8). From a cocktail fermented spontaneously at a pH of 3.6, a bacterial culture was isolated, which according to the API test seemed to be a variant of a *Lactobacillus plantarum* strain. This culture was able to produce lactic acid when fermentation was carried out at low pH values, but conditions for increased proliferation and lactic acid production of the strain must be further studied.

Keywords: lactic acid fermentation, vegetable cocktails, fermented vegetable cocktails

The solubilization of vegetables and fruits by macerating enzymes results in a fine dispersion liquid (ZETELAKI-HORVÁTH et al., 1984) containing almost all the valuable nutrients (protein, carbohydrates) (ZETELAKI-HORVÁTH & GÁTAI, 1977), vitamins (ZETELAKI-HORVÁTH & VAS, 1980), minerals (ZETELAKI-HORVÁTH et al., 1983) and dietary fiber (RIGÓ, 1984). These are of importance from the point of view of nutrition physiology since they are responsible for the normal physiological state of the human organism.

Vegetable cocktails prepared by macerating enzymes contain about 1% of dietary fiber. Dietary fiber is considered to play an important role in bile acid and cholesterol metabolism (STORY & KRITCHEVSKY, 1976) and to reduce blood glucose and the levels of two risk factors associated with heart disease (REISER, 1979).

In order to have the required shelf life, soft drinks must be heat-treated, which usually results in a considerable loss in their vitamin contents. To decrease the loss in vitamin C, either flash pasteurization (SZILÁGYI-TÓTH et al., 1985) or some other mild preservation method, such as lactic acid fermentation can be used.

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Lactic acid fermentation has been widely used for the preservation of various vegetables for centuries in the households of many countries. The original, mainly Gram-negative, bacterial flora of vegetables is replaced by a lactic flora in two days which improves storage stability (ANDERSSON, 1984). Lactic acid fermentation of vegetables is not only a preservation technique, resulting in products of long shelf life, but an efficient means of improving the sensory quality and the nutritive value of the vegetables as well. Thus, fermentation of vegetables decreases the nitrate content to about ten per cent of the original amount (ANDERSSON, 1986).

In the present work vegetable cocktails produced by enzyme technology (ZETELAKI-HORVÁTH et al., 1983; ZETELAKI-HORVÁTH, 1986) were fermented by lactic acid fermentation (ANDERSSON, 1986) in an attempt to obtain products with improved nutritional quality.

1. Materials and methods

1.1. Materials

1.1.1. Vegetables. For the vegetable cocktails beetroot, carrot, red paprika, horse radish and onion, obtained from the retail market, were used.

1.1.2. Enzyme preparation. For the enzyme treatment a macerating type of pectolytic enzyme (Rohament, P., Röhm & Haas, GFR) was used, with an activity of 2500 PGU mg^{-1}. One PGU (polygalacturonase unit) corresponds to the enzyme quantity which reduces the viscosity of 1 mg pectin in a standard pectin solution within 40 minutes, at 30 °C and pH 4 by 1 per $\eta_{sp} = 0.05$.

1.1.3. Starter cultures. For the fermentation of the enzyme treated vegetables *Lactobacillus plantarum,* a starter produced for the fermentation of vegetables in the Christian Hansen's Laboratory, Denmark, and four other starters for kefir, yogurt (LF-18), and cheese (HSA) and *Lactobacillus helveticus* (Swedish Dairy Association) were used. A culture isolated from vegetables, when fermented spontaneously at a low pH (3.6) was also used for fermentation.

1.2. Methods

1.2.1. Preparation of vegetables. Vegetables were washed (the beetroots and carrots were also peeled), then sliced by a vegetable slicer (Type: Assistant, Electrolux, Sweden) into 2×50 mm slices. Beetroot, carrot, red paprika, horse radish and onion were mixed in a ratio of 47 : 20 : 20 : 8 : 5. To 245 g of vegetables 255 cm^3 of water was added, then the whole mixture was homogenized in a homogenizer (Kenwood, M.F.G. Co. Ltd., England) for one minute. After homogenization the pH of the vegetable suspensions were adjusted with citric acid to the desired value.

1.2.2. Enzyme treatment and fermentation. The enzyme preparation of Rohament, P. (Rohm & Haas, GFR) was used in a concentration of 0.8% (w/w of the vegetables) and starter cultures in about 10^6 cells per cm^3 concentration. Reaction mixtures were then filled into 500 cm^3 flasks and incubated on a reciprocal shaker at a speed of 120 r.p.m. (stroke: 2.5 cm) for 22 hours at a temperature of 30 °C. Enzyme treatment and fermentation were carried over during the same period. Vegetable suspensions were homogenized after enzyme treatment for 2 minutes in an Ultra-Turrax apparatus.

1.2.3. Analysis of the vegetable cocktails: pH-measurement. The pH was determined by a radiometer (Type: PHM 62, Copenhagen).

Viscosity measurement. Viscosity was measured by testing the flow time of the different samples through a 20 cm^3 volumetric pipette with an inner diameter of 2 mm at the tip. The flow time was measured 6 times, and the average value (t_{sample}) was used to calculate the relative viscosity of samples:

$$\eta_{rel} = \frac{t_{sample}}{t_{water}} \qquad \eta_{sp} = \eta_{rel} - 1$$

where t_{water} is the flow time of the water.

Absorbancy measurement. Absorbancy of the centrifuged and filtered (by Millipore filter, type: GS, Millipore SA, France) samples was measured at a wavelength of 627 nm using a spectrophotometer (Vitatron, Holland).

Determination of the acid content. Acid content of the samples was determined by high pressure liquid chromatography (HPLC) according to a previously described method (ANDERSSON & HEDLUND, 1983).

Determination of the bacterial count. Bacterial count was determined by the agar plate method with MRS (de Man, Rogosa, Sharpe) agar (Oxoid), which is selective for the detection of lactic acid bacteria.

Sensory evaluation. For sensory evaluation of the samples, colour, odour, taste and the consistency were tested by a panel of 6 judges. The maximum number of scores was 10 in each case.

Mathematical statistical evaluation. From the results of the different measurements standard deviations were calculated. The different samples were compared by *t* test. As a basis of comparison, the sample representing the best result was chosen.

2. Results

2.1. Effect of pH

The pH value of the reaction mixture is important from the point of view of enzyme treatment and bacterial growth as well. The optimal pH for enzyme treatment is in the pH range of 3.8–4.2, while for good proliferation of the bacteria a higher pH (5.0–5.5) is necessary. The effect of the pH on

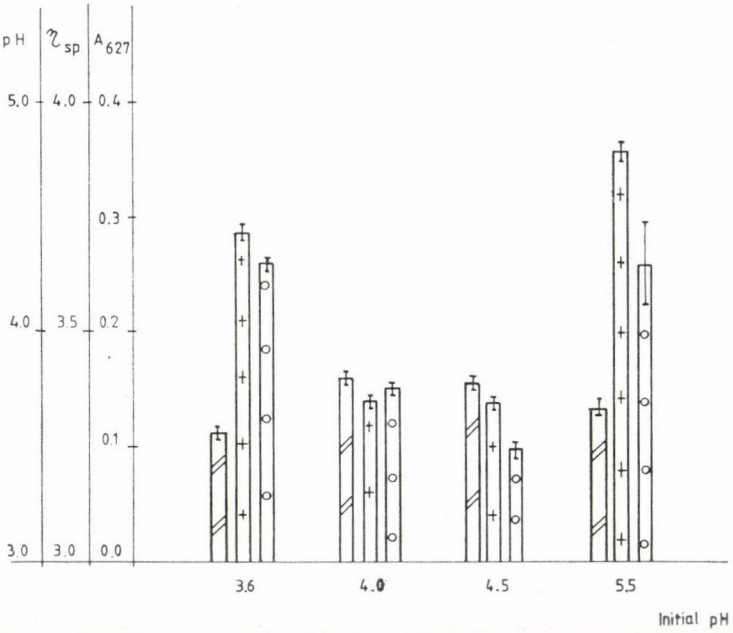

Fig. 1. pH (▨), specific viscosity (⊞) and absorbancy (⊡) of the vegetable cocktails fermented by *Lactobacillus plantarum* at various pH values

the fermentation of vegetables by *Lactobacillus plantarum* and the quality of cocktails was examined in the range of pH 3.6 to 5.5 (Fig. 1).

The decrease in the pH value of the samples after fermentation can be considered as a result of the lactic acid production of the starter culture. In the case of samples with lower initial pH values, almost non or a slight decrease in the pH was observed during fermentation. The higher the initial pH the greater was the decrease in the final value.

The highest viscosity was found in the samples of pH 5.5, without any pH adjustment, and the next highest in the samples of the lowest pH.

The absorbancy of the centrifuged and filtered samples was also the highest in the case of the lowest and the highest pH (Fig. 1).

No increase in the bacterial count was observed when the initial pH of the cocktails was 3.6, while at pH values of 4.0, 4.5 and 5.5 the bacterial count increased by about one and three orders of magnitude, respectively (Fig. 2).

In the course of organic acid determination by HPLC, no lactic acid production was found in the samples fermented at pH 3.6. Lactic acid production was very low at pH 4.0, while at pH values of 4.5 and 5.5 the lactic acid production was 0.22 and 0.46 %, respectively.

According to the sensory evaluation (Fig. 3), samples having the lowest initial pH proved to be the best, from the point of view of all the investigated

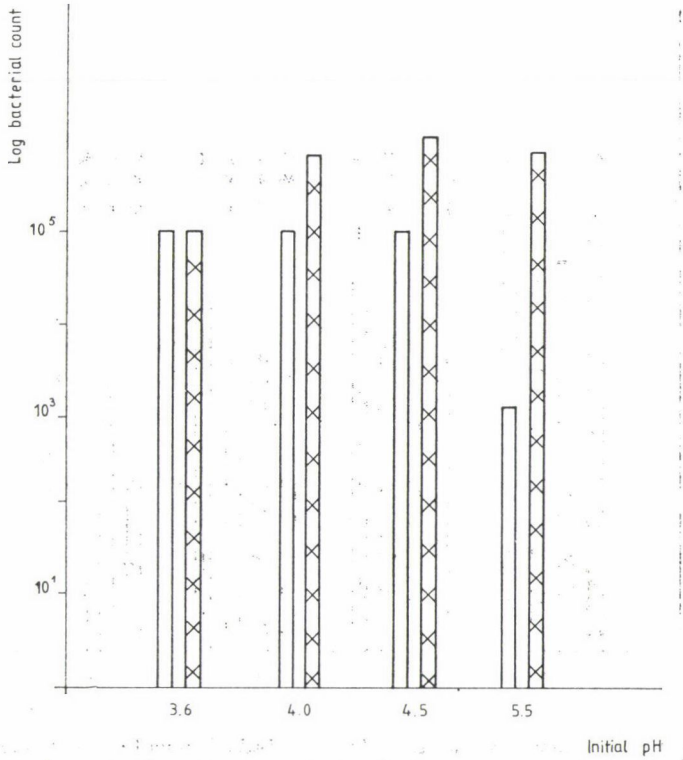

Fig. 2. Log. bacterial count of the cocktails fermented by *Lactobacillus plantarum* at various pH values. □: before fermentation, ⊠: after fermentation

parameters. The scores given to the colour, odour and taste of the samples, decreased with the increase of the initial pH. The differences in consistency of the samples of different initial pH values (Fig. 3) proved to be negligible.

2.2. Effect of starter culture

2.2.1. Fermentation at pH 4.5. The pH values of the vegetable cocktails were adjusted to pH 4.5 to ensure a condition which is not too unfavourable for both the enzyme and the starter culture. In this experiment, fermentation of beetroot cocktail without starter culture (spontaneous) was compared with fermentations where *Lactobacillus plantarum* and cultures for kefir, yogurt and cheese production were used.

After fermentation of the samples, the pH value was significantly the lowest in the case of *Lactobacillus plantarum*. The pH value of the other samples varied in a rather narrow pH range between 4.08 and 4.22.

The highest viscosity was measured in the samples fermented spontaneously and the lowest in the case of the yogurt culture. The absorbancy of

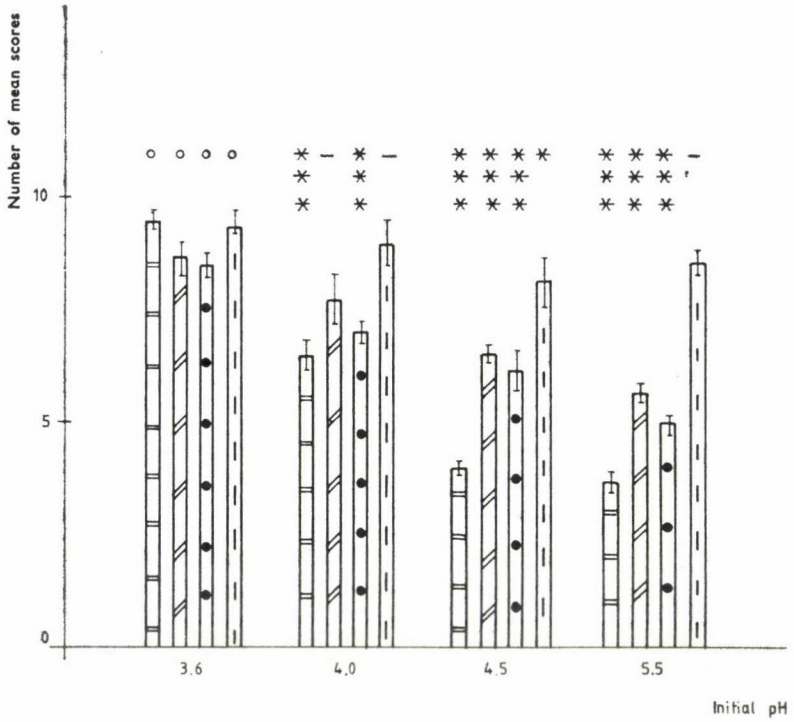

Fig. 3. Results of sensory evaluation of the cocktails fermented by *Lactobacillus plantarum* at various pH values. ▤: colour, ●: taste, ▨: odour, ▯: consistency. ○: basis of comparison; *: significant at 5% probability level; ** highly significant at 1% probability level; ***: very highly significant; at 0.1% probability level

the samples was rather low showing the lowest values in the spontaneously fermented cocktails (Fig. 4).

The highest rate of bacterial growth was found in the samples of spontaneous fermentation, where the bacterial number increased by more than four orders of magnitude. The number of bacteria increased by one order of magnitude in the samples fermented by *Lactobacillus plantarum* and the kefir culture, but no bacterial proliferation was found in the case of yogurt and cheese cultures (Fig. 5).

When comparing the acid production of the different cultures, the highest lactic acid production was measured in the spontaneously fermented samples (0.42%). Lactic acid production of the *Lactobacillus plantarum* and the kefir culture was very highly significantly lower (0.18 and 0.09%, resp.) than that of the spontaneous culture, while no lactic acid production was found in the cocktails fermented by the yogurt and cheese cultures.

According to the sensory evaluation, the colour of the samples fermented by the yogurt culture was found to be the best (Fig. 6). The odour was found the most pleasant in the case of spontaneous fermentation, while

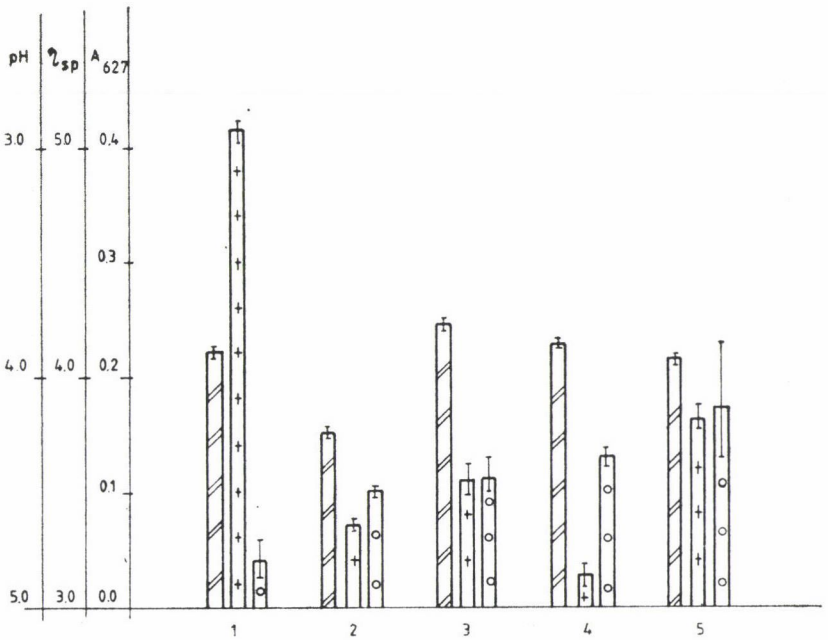

Fig. 4. The pH (▨), specific viscosity (⊞) and absorbancy (⊙) of the cocktails fermented by different starter cultures: 1. without starter culture, 2. *Lactobacillus plantarum*, 3. kefir, 4. yogurt, 5. cheese. Initial pH: 4.5

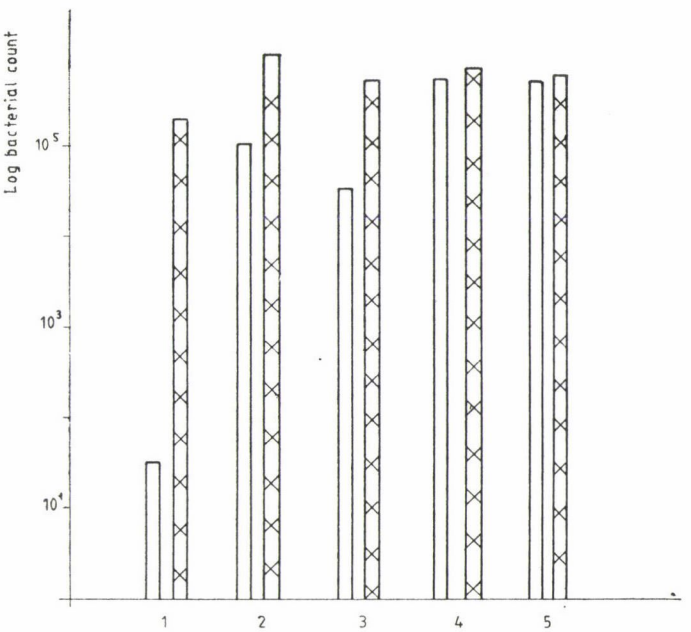

Fig. 5. Log. bacterial count of the cocktails fermented by different starter cultures: 1. without starter culture, 2. *Lactobacillus plantarum*, 3. kefir, 4. yogurt 5. cheese. □: before fermentation, ⊠: after fermentation

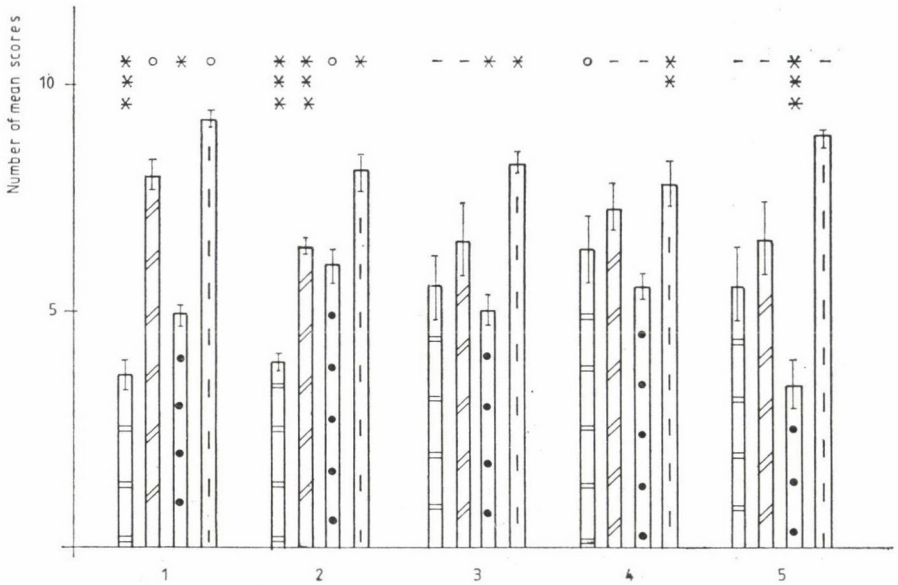

Fig. 6. Results of sensory evaluation of the cocktails fermented by different starter cultures: 1. without starter culture, 2. *Lactobacillus plantarum*, 3. kefir, 4 yogurt, 5. cheese. ⊟: colour, ▨: odour, ●: taste, ▯: consistency, ○: basis of comparison, *: significant, **: highly significant, ***: very highly significant

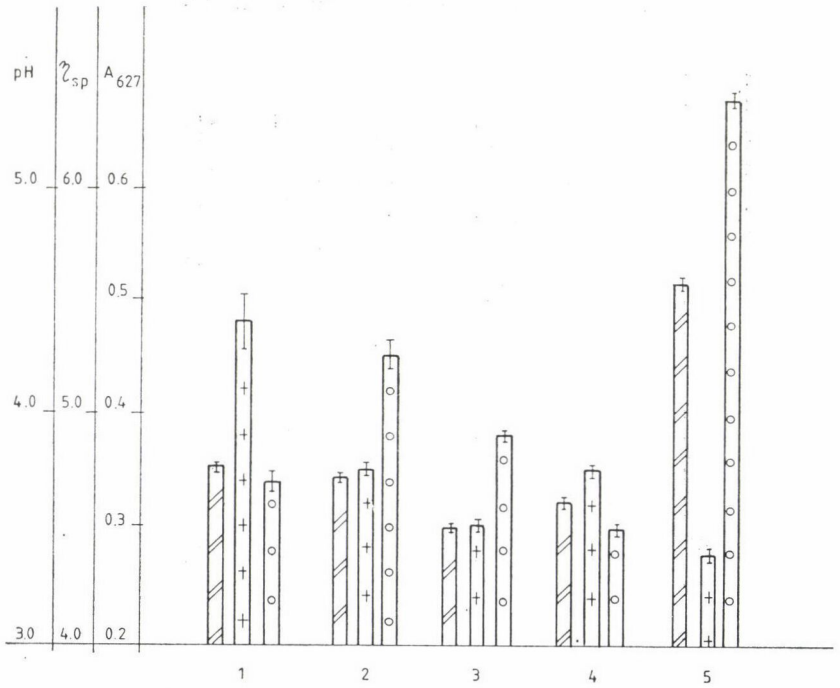

Fig. 7. The pH (▨), specific viscosity (⊞) and absorbancy (◎) values of the cocktails fermented spontaneously and by various starter cultures: 1. *Lactobacillus plantarum*, 2. *Streptococcus lactis*, 3. isolated culture, 4–5. without starter culture. Initial pH at 1–4: 3.8, at 5: 5.5

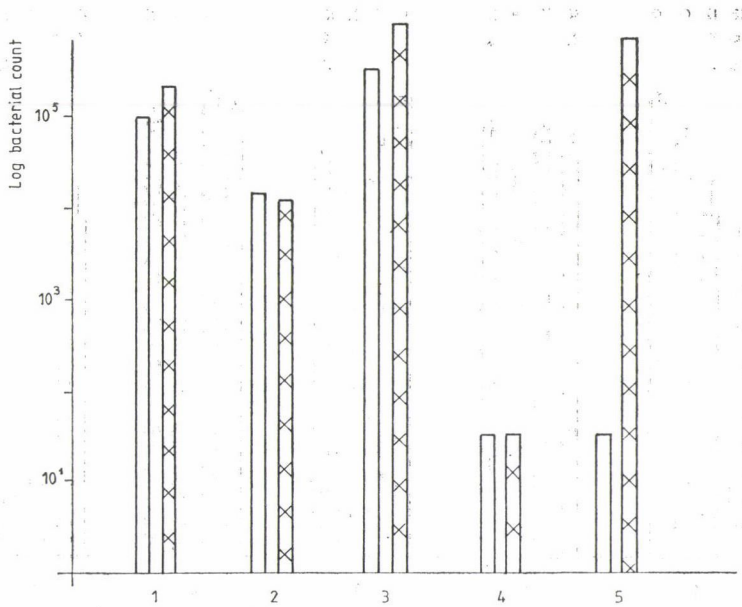

Fig. 8. Log. bacterial count of the cocktails fermented spontaneously and by different starter cultures: 1. *Lactobacillus plantarum*, 2. *Streptococcus lactis*, 3. isolated cultures, 4–5. without starter culture. □: before fermentation, ⊠: after fermentation. Initial pH at 1–4: 3.8, at 5: 5.5

the taste of the cocktails proved to be the best when *Lactobacillus plantarum* was used (Fig. 6). The taste of the latter was significantly, highly significantly and very highly significantly better than the taste of those fermented spontaneously, or by the other cultures.

2.2.2. Fermentation at pH 3.8. In the course of the following experiment enzyme treatments and the fermentations were carried out at pH 3.8. As starter cultures *Lactobacillus plantarum, Streptococcus lactis* and a culture isolated (from samples, fermented spontaneously at a pH of 3.6) were used. Spontaneous fermentations were also started without pH adjustment (pH 5.5) and at a pH value of 3.8.

The lowest pH value was measured in the cocktails fermented by the isolated culture at pH 3.8 and in the spontaneously fermented cocktails (at pH 5.5) after fermentation (Fig. 7). The viscosity of the *Lactobacillus plantarum* fermented vegetable cocktails proved to be very highly significantly the highest.

The absorbancy of the centrifuged and filtered samples was very highly significantly the highest in the spontaneously fermented samples without any pH adjustment. This was followed by the values of samples fermented by *Streptococcus lactis* (Fig. 7).

After fermentation a slight increase was found in the bacterial count of the samples fermented by *Lactobacillus plantarum* and the isolated culture

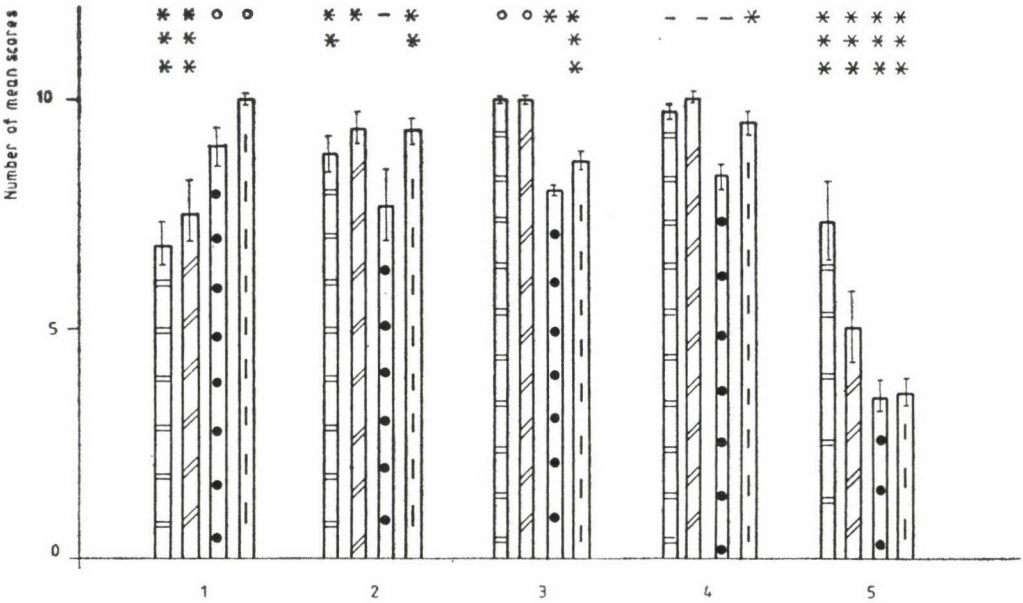

Fig. 9. Results of sensory evaluation of the cocktails fermented by different starter cultures: 1. *Lactobacillus plantarum*, 2. *Streptococcus lactis*, 3. isolated cultures, 4–5. spontaneous fermentation. ▭: colour, ▨: odour, ◆: taste, ▯: consistency, o: basis of comparison, *: significant, at 5% probability level; **: highly significant at 1% probability level; ***: very highly significant at 0.1% probability level. Initial pH at 1–4: 3.8, at 5: 5.5

(Fig. 8). A very highly significant increase occurred in the bacterial count of the samples spontaneously fermented without any pH-adjustment, while in samples fermented spontaneously at pH 3.8 no bacterial growth was found. In the samples fermented by *Streptococcus lactis* no increase was found in the bacterial count during the fermentation (Fig. 8).

No lactic acid production was detected in the cocktails fermented by *Lactobacillus plantarum* and *Streptococcus lactis* nor in those fermented spontaneously. In samples fermented by the isolated culture at pH 3.8 and 5.5, a lactic acid production of 0.19 and 0.52%, respectively, was found.

In the course of sensory evaluation the taste proved to be the best in the samples fermented by *Lactobacillus plantarum*, followed in decreasing order by those fermented spontaneously (at pH 3.8), by the isolated culture and by *Streptococcus lactis*.

The consistency proved to be highly, very highly significantly and significantly better in the samples fermented by *Lactobacillus plantarum* than in those fermented by *Streptococcus lactis;* in the isolated culture and in those fermented spontaneously at pH 3.8, respectively.

The samples, fermented spontaneously at pH 5.5, were found to be very highly significantly the most unfavourable from the point of view of all the examined properties (Fig. 9).

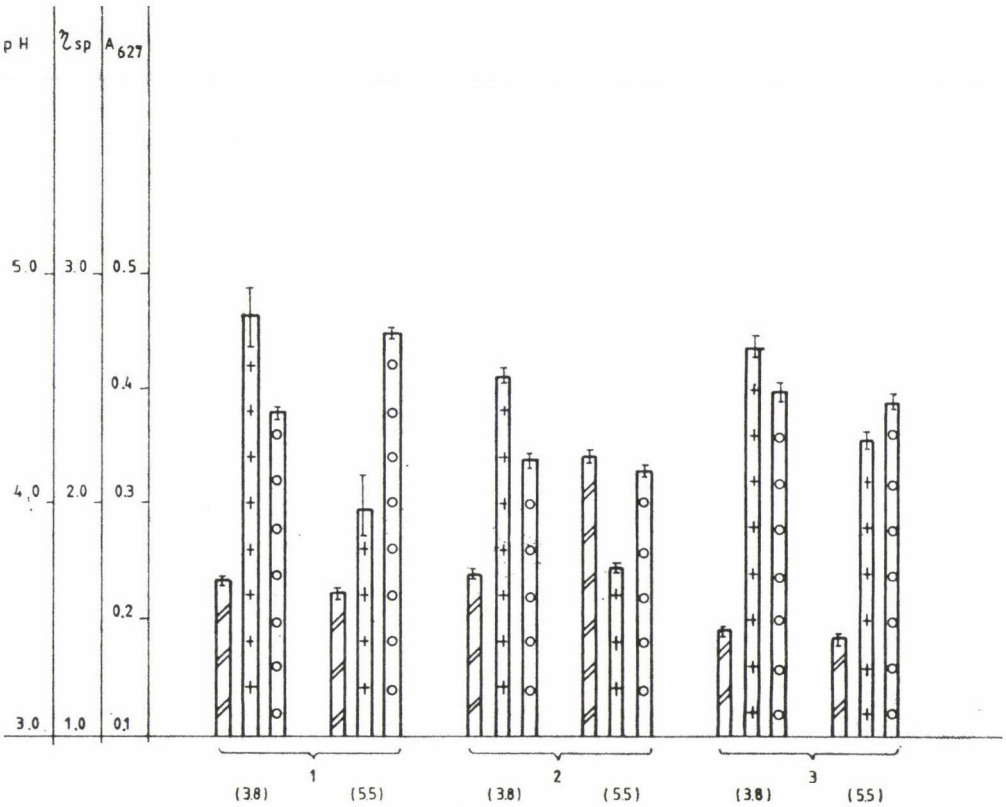

Fig. 10. The pH (▨), specific viscosity (⊞) and absorbancy (◎) of the cocktails fermented at pH 3.8 and 5.5 by different starter cultures: 1. *Lactobacillus plantarum*, 2. *Lactobacillus helveticus*, 3. isolated culture. Fermentation at 30 °C

Fermentation of beetroot cocktails with and without pH adjustment was carried out by *Lactobacillus helveticus* and was compared with samples fermented by *Lactobacillus plantarum* and the isolated culture.

The pH value was found to be the lowest after fermentation of the cocktails by the isolated culture with and without pH adjustment and the highest, when *Lactobacillus helveticus* was used.

The viscosity of the cocktails and the absorbancy of the filtered serum was the highest in the case of *Lactobacillus plantarum*, followed by that of the isolated culture (Fig. 10).

Samples fermented by the three cultures at pH 5.5 without pH adjustment, were found unfavourable from the point of view of all the three examined parameters.

In the samples fermented by *Lactobacillus plantarum* and the isolated culture at pH 3.8 and at pH 5.5 the bacterial count increased by about one and a half and two orders of magnitude. The highest growth was found in

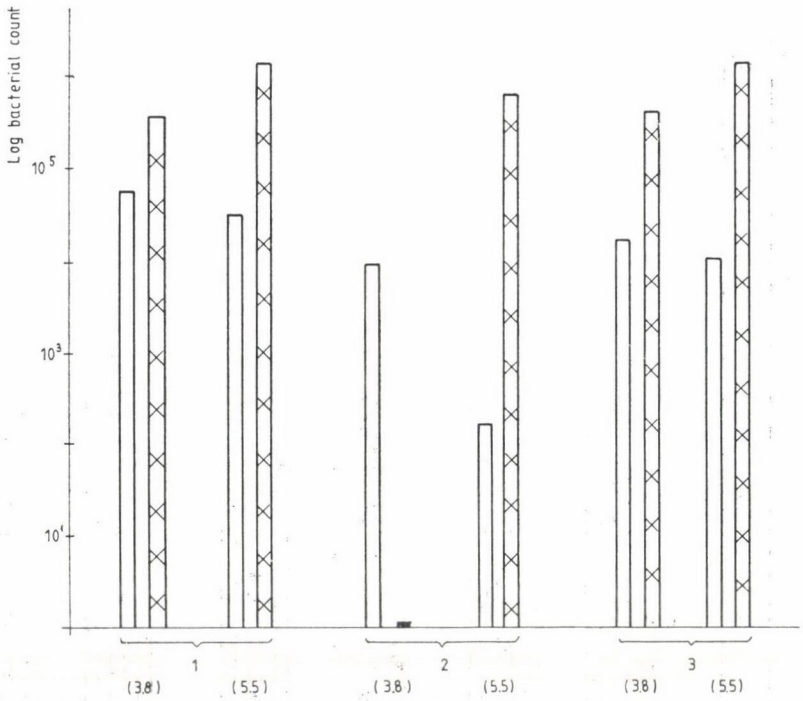

Fig. 11. Log. bacterial count of the cocktails fermented at pH 3.8 and 5.5 by different starter cultures: 1. *Lactobacillus plantarum*, 2. *Lactobacillus helveticus*, 3. isolated culture, 0.05 g 500 cm^{-3}. □: before fermentation, ☒: after fermentation

the cocktails fermented by *Lactobacillus helveticus* and the isolated culture at a pH of 5.5. At pH 3.8 the number of viable bacteria decreased very highly significantly (Fig. 11).

Lactic acid production in the cocktails fermented by *Lactobacillus plantarum* and *Lactobacillus helveticus* and the isolated culture at pH 5.5 proved to be 0.6, 0.1 and 0.78 %, respectively. At pH 3.8 lactic acid production was found only in the cocktails fermented by the isolated culture.

In the course of the organoleptic evaluation the colour and taste were found significantly and very highly significantly better in the samples fermented by the isolated culture at pH 3.8 than those fermented by *Lactobacillus plantarum* and *Lactobacillus helveticus*. The colour, consistency and taste of the samples fermented at pH 5.5 proved to be very highly significantly less pleasant than of those fermented at pH 3.8 (Fig. 12).

2.3. Identification of the isolated culture

From the beetroot cocktails in which a good bacterial growth was observed after spontaneous fermentation at pH 3.6, cultures were isolated on MRS agar. After subculturing in MRS broth at 30 °C for 24 h on a reciprocal

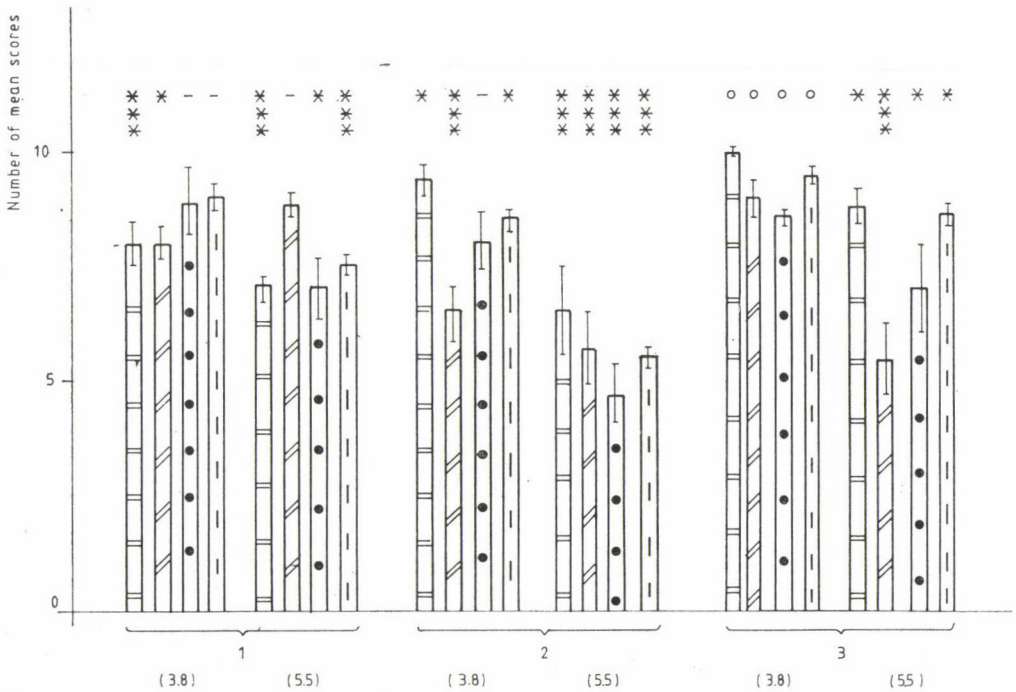

Fig. 12. Results of sensory evaluation fermented at pH 3.8 and 5.5 by different starter cultures: 1. *Lactobacillus plantarum*, 2. *Lactobacillus helveticus*, 3. isolated culture. ▭: colour, ▱: odour, ◉: taste, ▯: consistency, ○: basis of comparison, *: significant at 5% probability level; **: highly significant at 1% probability level; ***: very highly significant at 0.1% probability level

shaker API tests were carried out with the use of API 50 HCl medium, which enables the fermentation of 49 carbohydrates on the API 50 CH gallery. Microorganisms, suspended into the medium were inoculated into each gallery. During incubation colour changes of the indicator occurred, as a result of the fermentation of sugars into acids.

According to the results, the biochemical profile of the isolated strains is very similar to that of *Lactobacillus plantarum* with the difference that the isolated strain is unable to utilize melibiose, D-turanose and L-fucose.

The morphology of the isolated micro-organism on MRS agar proved to be identical with that of *Lactobacillus plantarum*.

3. Conclusions

Enzymatic solubilization of vegetables results in fibrous soft drinks with increased nutritional value. Their nutritive value was intended to be increased further by lactic acid fermentation. It is not indifferent which isomer

of the lactic acid is available in the diet, as L (+) lactic acid can be utilized during human metabolism, while its D (−) form is excreted or oxidized in the liver (KANDLER & STETTER, 1977). As Lactobacilli are considered to produce L (+) lactic acid, their use for the fermentation of vegetables should be more advantageous.

It can be concluded from our results that the enzyme treatment and the lactic acid fermentation of the vegetables can be carried out at the same time.

From the point of view of the organoleptic properties of the final products (cocktails), the adjusted lowest pH proved to be the best in the course of enzyme treatment and the fermentation.

No correlation was found between the purple colour of the whole sample and the absorbancy of the serum. When the absorbancy of the serum samples was high, the colour of the whole cocktails was often slightly brown. Cocktails having higher pH than 3.8 showed no pH change after fermentation, but after 24 and 28 hours they turned brown even in a dark room.

Among the tested pH values, pH 5.5 proved to be the most advantageous for lactic acid production by the applied strains.

The highest lactic acid production was 0.76 g 100 cm^{-3} in the samples fermented by the isolated culture at pH 5.5, which was rather good in a short (22 h) fermentation period. When the fermentation was carried out at pH 3.8 the lactic acid production of the isolated strain was only 0.23 g 100 cm^{-3}, while in samples fermented by other bacteria, no lactic acid production was found.

On considering the applied strains, organoleptic properties of the vegetable cocktails proved to be the best when the cultures used were *Lactobacillus plantarum* (Christian Hansen's strain) and the strain isolated in the present work.

According to the API test, the isolated culture is considered to be *Lactobacillus plantarum*.

In the future, our work will be continued with strain improvement and isolation of other strains capable of growing rapidly, and the determination of those parameters which result in high production of lactic acid in the L (+) form.

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HYDROGEN ION TITRATION BEHAVIOUR OF PROTEIN CARMIN FROM SAFFLOWER SEED (*CARTHAMUS TINCTORIUS* L.)

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The hydrogen ion titration curve of the high molecular weight protein, carmin from safflower seed indicates that the side chain carboxyl, imidazole and ε-amino groups titrated with normal pK_{int} values. Their number agreed well with the analytical values obtained from amino acid analysis. However, tyrosyl phenolic groups had an abnormal $pK_{int} = 10.75$. The increase in the value of the electrostatic interaction parameter is discussed with the available data on the structural changes in the protein moiety.

Keywords: hydrogen ion binding, equilibrium constant, hydrophobicity, conformation

Safflower seed (*Carthamus tinctorius* L.) is an oil seed and is grown for its oil mainly in India and also in North America. The total proteins from the deoiled seed has been extracted, and studied in detail for its various properties and characterized (LATHA & PRAKASH, 1984). Carmin, the high molecular weight protein fraction from the safflower seed is isolated to homogeneity and its hydrodynamic properties investigated (LATHA & PRAKASH, 1985). The protein has been shown to have a sedimentation coefficient of 12.75, a molecular weight of ~260 000, rich in aperiodic structure and globular shaped (LATHA & PRAKASH, 1986). It has become evident from the dissociation and denaturation studies (PRAKASH, 1986) that the binding of H^+ is of considerable importance in the stability of the quaternary structure of carmin.

In this study we have titrated the native carmin with H^+ and the titration curve is discussed in terms of the structural changes and amino acid composition.

1. Materials and methods

Safflower seeds of the variety A-1 were obtained from the Karnataka State Seed Corporation Ltd. (Bangalore, India). They were flaked and dried in a cabinet air drier at 60 °C for 3 h. The dried flakes were defatted with *n*-hexane and were air-dried to remove the residual solvent. The hulls were

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separated by differential sieving and passed through a plate mill and subsequently through a 60 mesh sieve. The resultant flour had less than 1% fat.

1.1. Isolation of carmin

The total proteins from safflower seed flour was extracted in 0.01 mol phosphate buffer of pH 7.5 containing 1 mol NaCl using a 1 : 10 flour to solvent ratio. The slurry was centrifuged at 10 000 g for 30 min at 20 °C in a Hitachi 55P preparative ultracentrifuge. To the supernatant obtained solid powdered ammonium sulfate was added slowly under constant stirring so that the final concentration of ammonium sulfate was 10.4%. The suspension was kept in cold for 90 min and the precipitated protein was separated by centrifugation at 10 000 g for 30 min at 20 °C. The precipitate was dissolved in the extraction buffer and the process of ammonium sulfate precipitation (10.4%) repeated. The final precipitate obtained was made free of salt by dialysing versus distilled water and lyophilized.

1.2. Protein concentration

The concentration of carmin was determined by macro-Kjeldhal procedure (AOAC, 1984). A calibration curve relating the milligrams of nitrogen present in the protein sample to the ultraviolet absorbance of the protein at 280 nm was obtained for routine determination of protein concentration. The absorption coefficient $E_{280\,nm}^{1\%,\,1\,cm}$ gave a value of 11.2 for carmin and was used n all the calculations of protein concentration.

1.3. Ultraviolet absorption spectrum

The ultraviolet absorption spectrum of the protein was recorded using a Perkin Elmer 124 double beam spectrophotometer in the range of 400–240 nm in a 1 cm quartz cell at room temperature (~25 °C).

1.4. Hydrogen ion titration

The pH measurements were made at 20 ± 0.05 °C with a Radiometer pH meter TTT-2, and Orion Research Microprocessor Ionanalyser 901. The pH meter was standardised with standard buffers of p 4.01, 6.50 and 9.05. All solutions were prepared in deionised water. The hydrogen ion titration was conducted at 20 °C in 0.5 mol KCl solution. To 10 cm^3 of 1% protein solution in 0.5 mol KCl, enough standard HCl solution was added to bring the pH to 2.0. Carbonate free KOH solution was slowly added in increments of 10 μl with an Agla or Hamilton micrometer syringe and after each addition, the pH of the solution was noted, after thorough mixing and stabilization of pH meter readings. Titration was carried out upto pH 12, flushing nitrogen above pH 6. A blank titration was carried out with 10 cm^3 of 0.5 mol KCl in a

similar fashion as described above for the protein solution. From the difference in pH values, the number of hydrogen ions bound/dissociated per molecular weight of 100 000 for the protein was calculated.

1.5. Spectrophotometric titration

This was carried out by adjusting the pH of the protein solution in buffer with 1 mol sodium hydroxide to different pHs and measuring the absorbance at 295 nm in a Perkin-Elmer 124 spectrophotometer (TANFORD, 1962).

2. Results and discussion

2.1. Potentiometric titrations

The hydrogen ion titration curve of carmin as determined in 0.5 mol KCl solution is shown in Fig. 1. Titrations commenced at pH ~7.0 and the protein was soluble over the total pH range except for pH 5.0–3.0. Precipitation of the protein occurred at pH 5.0 and redissolved only when the pH was lower than 3.0.

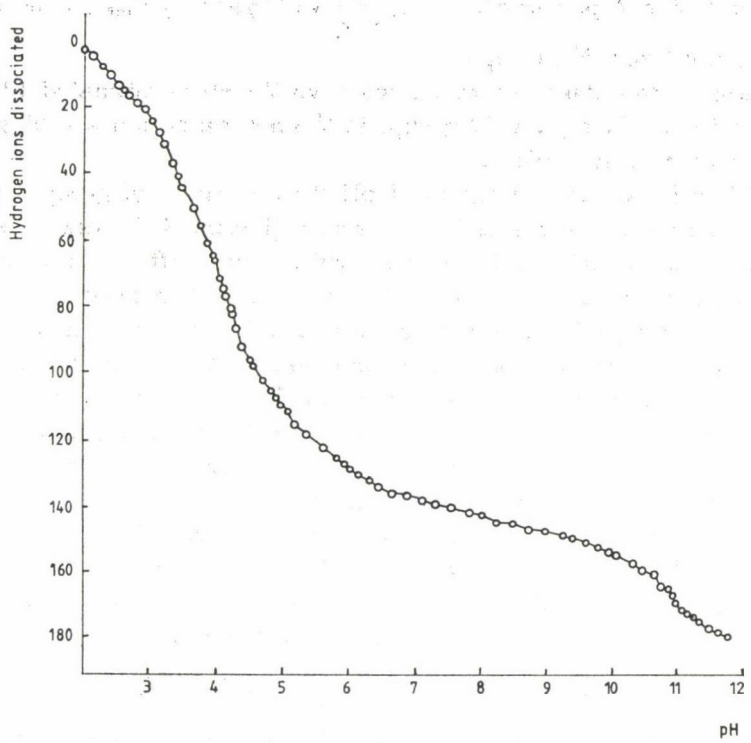

Fig. 1. Hydrogen ion titration curve of carmin at ionic strength of 0.5 and at 20 ± 0.05 °C; o: experimental data points; ——: calculated curve. The curve drawn is the mean value ($\bar{x}$) of three (n = 3) measurements. The size of the "circles" indicates the error bars

Counting of groups was made from the point of maximum proton charge in the region pH ~2.0 where the curve approaches a plateau region as described by TANFORD (1962). Calculations were based on a molecular weight of 100 000 for the protein. At the acid end point (pH 2) a maximum of 135 hydrogen ions were bound. Using this as the reference point, the number of hydrogen ions dissociated (r) was estimated as a function of pH. In the plot of r vs pH (Fig. 1) the curve can be divided into three regions, acidic (pH 2 to 6), neutral (pH 6 to 8) and alkaline (above pH 8).

The analyses of the titration curves were performed by the method developed by Linderstrøm—Lang and described by TANFORD (1962), using the relation

$$\mathrm{pH} - \log \frac{r_i}{n - r_i} = \mathrm{p}K_{int}^{(i)} - 0.868\, WZ \qquad (1)$$

where r_i is the average degree of dissociation of a titratable group i, whose equilibrium constant is $K_{int}^{(i)}$ which mainly depends on the size of the protein and ionic strength, Z is the net charge on the protein, and W is the electrostatic interaction factor. A plot of $\mathrm{pH} - \log \frac{r_i}{n - r_i}$ vs Z yields $\mathrm{p}K_{int}$ as the intercept at $Z = 0$, and 0.868 W as slope.

Based on the above equations we have therefore estimated 135 groups titrated in the acidic region, 17 groups in the neutral region and 37 groups in the alkaline region upto pH 12.0.

2.1.1. pH 2–6: In the region of pH 2 to 6, carboxyl groups dissociate. From the amino acid composition of carmin (LATHA & PRAKASH, 1986) the total number of aspartic and glutamic acid residues, after correction for the number of amide groups, was 140 ± 6. This error, in the determination of the total number of aspartic and glutamic acid residues arises mainly due to the uncertainty in estimation of amide ammonia in an automatic amino acid analyzer. It is reported that some ammonia is liberated due to the destruction of serine and threonine (SPAHR & EDSALL, 1964). However, no such corrections were applied for the calculation of amide ammonia. This is likely to contribute to a slightly high amide ammonia value as a result of the content of aspartic and glutamic acids will be low. In all the calculations no correction for any possible anion binding was made (TANFORD, 1962).

Using the values of n from 133 to 140, the calculations were made. Initially, the net charge on the protein, Z was taken as equal to the number of hydrogen ions bound. A plot of $\mathrm{pH} - \log \frac{r}{n - r}$ vs Z for $n = 135$ is shown in Fig. 2. A linear regression line was fit for the experimental data points giving an intercept at Z of 4.33 and a slope of 0.0061 was obtained. Using these values of $\mathrm{p}K_{int}$ 0.868 W and n, r, the number of hydrogen ions dissociated

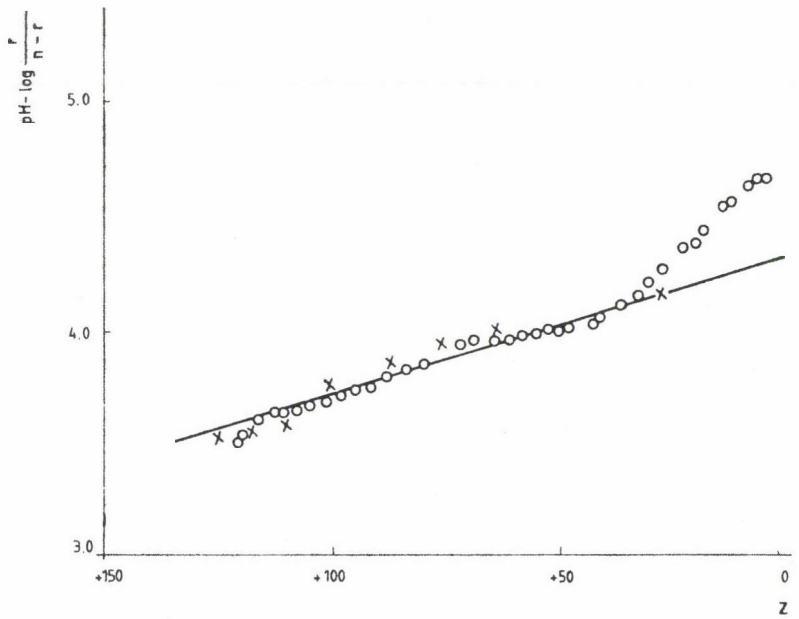

Fig. 2. Plot of pH $-$ $\log \frac{r}{n-r}$ r vs Z for titration of side-chain carboxyl groups. ×——× indicate calculated points after curve fitting. o——o experimental data points. The correlation coefficient (r^2) calculated is 0.967. Z values (net charge on the protein) in the range $+30$ to $+115$ are utilized for caleulations

was calculated with the Linderstrøm—Lang equation. The calculated curve is shown as solid line in Fig. 1. These calculations show excellent agreement between the experimental data and the calculated data points. The value of n from the titration data agreed well with the amino acid composition data. The pK_{int} value of 4.33 for the side-chain carboxyl groups was well within the range of values of 4.0–4.8 for many proteins as reported by TANFORD (1962). The values of pK_{int} for carboxyl groups in the case of groundnut protein conarachin as reported by SHETTY & NARASINGA RAO (1977) is 4.96 and for glycinin as reported by CATSIMPOOLAS and co-workers (1971) is 4.3 are note worthy. Since the α-carboxyl and α-amino groups represent a very small portion of the total groups titrated, their pK_{int} values cannot usually be derived from experiment (TANFORD, 1962). Thus, the carboxyl dissociation did not exhibit any abnormal features (Table 1). This clearly implies that most of the titratable groups are available at the surface of the protein itself, and they are able to accept or release hydrogen ions in this location without requiring any modification of the protein conformation in the vicinity of the titratable group. Since carmin has been shown to be globular protein, tightly folded so as to exclude solvent from most of the interior portions, the titration groups must be nearly always at the surface.

Table 1

Titratable groups and their characteristics in carmin at 20 ± 0.05 °C and in 0.5 ionic strength

Groups	n[a] from titration	n[b] from amino acid analysis	pK_{int}[c]	W[d]
Carboxyl	135	140 ± 6	4.33 ± 0.02	0.007 ± 0.001
Imidazole	17 ± 1	17 ± 2	7.63 ± 0.05	0.06 ± 0.003
ε-amino	17 ± 1	17 ± 1	9.13 ± 0.03	0.02 ± 0.005
Phenol	20 ± 1	20 ± 1	10.75 ± 0.07	0.06 ± 0.004

[a] The reported value of n is an average of three experiments from where the curves were drawn and data analysed. The error bars shown is the variation maximum one observes between the three measurements.

[b] The reported value of n is average of two amino acid analyses. The error bars shown is the variation one gets between the two experiments in the calculation of number of residues of amino acids.

[c] The pK_{int} value is calculated as the intercept at zero Z value. The mean value and the σ of intercept are the values indicated.

[d] The W value is calculated from the slope of the Figures 2, 3, 4 and 5 which is equal to 0.868 W. The mean value and the σ of W obtained from such a result, are indicated.

2.1.2. pH 6–8: In the neutral pH region, there is overlapping due to the dissociation of both carboxyl and imidazole groups. Hence from the experimental r values, r due to carboxyl dissociation using $pK_{int} = 4.33$ was deducted and the corresponding r values due to imidazole dissociation only were obtained. With these values and $n = 17$ for imidazole, plot of pH–log r per $n - r$ vs Z was made (Fig. 3). Values of $pK_{int} = 7.63$ and $0.868\ W = 0.06$ were obtained (Table 1). The pK_{int} expected for normal imidazole groups is 7.0 (TANFORD, 1962). The value obtained by us is higher, possibly indicating that the imidazole groups are in a more hydrophobic environment. The protein has a hydrophobicity index of 824 cals per residue (PRAKASH & NARASINGA RAO, 1986).

2.1.3. Above pH 8.0: Normally, tyrosyl dissociation is followed independently by spectrophotometric titration. With increase in pH from 8 to 11 dissociation of phenolic groups takes place leading to increased absorbance and shift in the absorption maximum from 280 nm to 293–295 nm for tyrosine residue. Hence the dissociation of tyrosyl residue is followed by measurement of absorbance at 295 nm as a function of pH (TANFORD, 1955). The calculation of molar extinction change upon increasing the pH from neutral to alkaline conditions corresponds to 20 tyrosine residues per mole of protein (Fig. 4). Application of the Linderstrøm–Lang relation to the titration data yielded a value of $pK_{int} = 10.75$ for the tyrosine group (Table 1). This value is higher as compared to the value of 9.6 for a normal phenolic group, though values of the magnitude obtained in this investigation are not uncommon with many proteins (TANFORD, 1962) since tyrosine is a hydrophobic amino

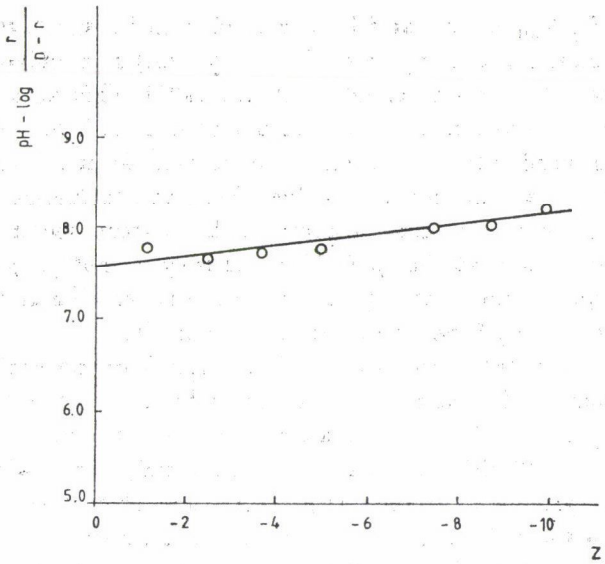

Fig. 3. Plot of pH $-$ log $\frac{r}{n-r}$ vs Z for titration of imidazole groups. The correlation coefficient (r^2) calculated for this plot is 0.91. Z values in the range of -1 to -10 are utilized for calculations

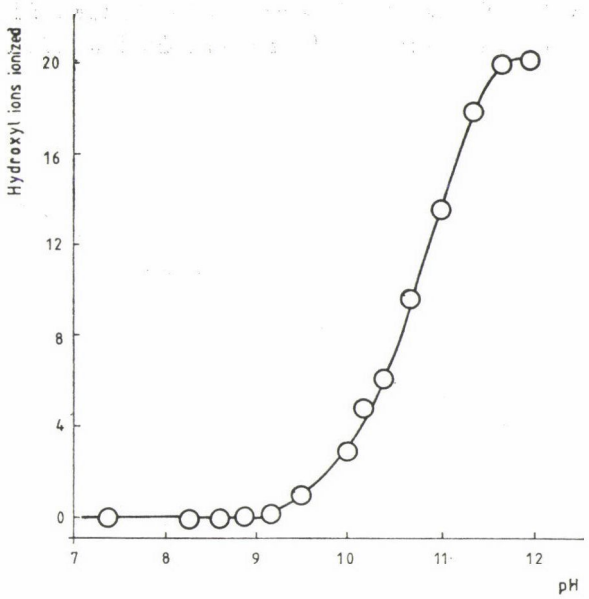

Fig. 4. Plot of hydroxyl ions ionized as a function of pH as determined spectrophotometrically. The curve drawn is the mean value ($\bar{x}$) of (n = 3) three measurements. The size of the "circles" indicates the error bars

acid. A value of $pK_{int} = 10.5$ and 11.3 were obtained respectively for conarachin (from groundnut) and glycinin (from soybean) respectively (SHETTY & NARASINGA RAO, 1977; CATSIMPOOLAS et al., 1971). This abnormal pK_{int} of phenolic residues is due largely to the fact that although the titratable parts must project outward into the solvent to be titratable, it is possible that the extent to which they can free themselves from the influence of the hydrophobic region depends on many factors such as stereospecificity and conformation of the protein at that pH. Hence if they do not project sufficiently far, the unchanged form of the group will become stabilized relative to the charged form, and the pK will be altered accordingly.

Knowing the total content of basic groups from potentiometric titrations, we estimated the lysine content by subtracting the tyrosyl groups obtained from spectrophotometric titrations. This yields a value of $pK_{int} = 9.13$ and $0.868\ W = 0.02$ (Fig. 5) (Table 1). The pK_{int} was within the range reported for many proteins (TANFORD, 1962). The value of W was lower than that obtained in the acid and neutral ranges of titration of carmin.

At this stage, using the values of n, pK_{int} and W for carboxyl, imidazole and amino groups given in Table 1, the titration curve was calculated from pH 2 to 11.5. The calculated titration curve agreed with the experimental data points within experimental error (Fig. 1).

From Table 1, it is apparent that the value of W steadily increases from acid pH to neutral pH from a value of 0.007 to 0.06 and in the alkaline region reaches a value of 0.02. This low value of W in the acid region may be due to dissociation and denaturation of carmin in this low pH range (PRAKASH,

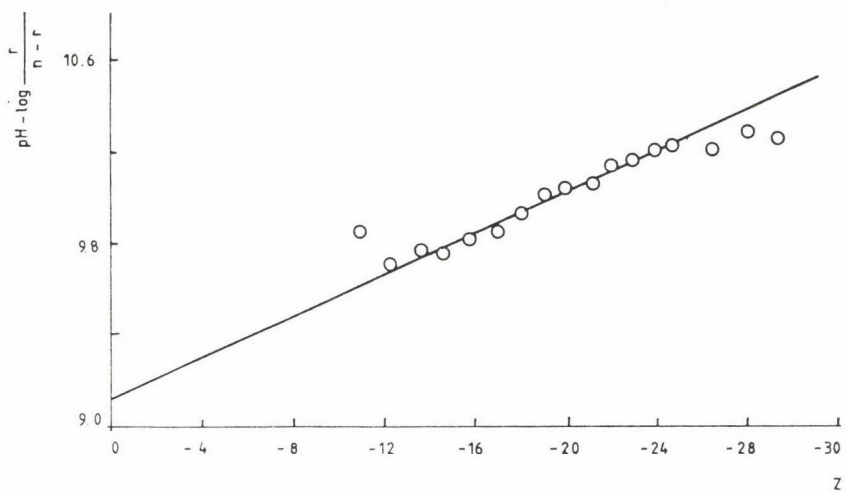

Fig. 5. Plot of $pH - \log \frac{r}{n-r}$ vs Z for titration of ε-amino groups. The correlation coefficient (r^2) calculated is 0.988. Z values in the range -11 to -28 are utilized for calculations

1986b). Evidence of conformational change to a more extended structure at low pH (pH ~2.5) is available (PRAKASH, 1986b). This may be responsible for the low value of W in the acidic range of pH.

3. Conclusion

The above results indicate that carmin, the high molecular weight protein fraction from safflower seed has normal pK_{int} values for the side chain carboxyl, imidazole and ε-amino groups. However, the tyrosyl phenolic groups had an abnormal pK_{int} of 10.75. The values observed are in conformity with other high molecular weight oilseed proteins (CATSIMPOOLAS et al., 1971; PRAKASH & NARASINGA RAO, 1986). However, these values have a high bearing on the association-dissociation and denaturation behaviour of carmin in acidic and alkaline solutions. These results clearly point out that most of the charged groups are available on the surface of the protein and can be easily titrated whereas the hydrophobic groups such as tyrosyl residues are imbedded in the protein or are present in hydrophobic pockets such as subunit contact areas similar to many standard proteins described by TANFORD (1955).

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ANALYSIS OF VOLATILE COMPONENTS IN VALENCIA ORANGE JUICE FROM CUBA

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Volatile flavour compounds present in the recovered condensate from commercial orange juice concentration were extracted with diethyl ether. The α,β-unsaturated aldehydes, aliphatic aldehydes, methyl ketones, alcohols, esters, acids and amines were systematically separated by chromatographic and chemical methods. Compound identification was made by comparison of relative retention data, authentic reference compounds, gas and thin-layer chromatography, coupled gas chromatography-mass spectrometry and spectroscopic techniques. Seventy-one compounds were identified including the following new compounds: 2-hexanol, β-terpineol, *n*-tetradecanal and tentatively C_7 to C_9, C_{11}, C_{12}, C_{14} and C_{16} carboxylic acids.

Keywords: volatile flavour components, orange juice volatiles

Investigations concerning the flavour of orange juice have required a workable knowledge of the compounds contributing to the flavour. Although the ultimate criteria of acceptability for citrus products lie in flavour appeal as determined by sensory tests, the development of such knowledge using instrumental and chemical methods has been necessary.

In the analytical studies of the orange juice flavour, different materials such as natural juice and recovered essences have served as good sources for those studies (PINO & TORRICELLA, 1983). Different aids for separation and identification of the individual components have also been reported. The most commonly used methods have been coupled GC-MS (SCHULTZ et al., 1964; SCHULTZ et al., 1967), column chromatography (KIRCHNER & MILLER, 1957; SCHREIER et al., 1977) and some conventional chemical methods (ATTAWAY et al., 1962; ATTAWAY et al., 1964). Preliminary fractionation of the complex mixture of volatile components can greatly simplify subsequent identification, particularly if it is possible to separate according to the functional group class. This type of technique has not been used widely in the analysis of the volatile flavour compounds in processed orange products. Nevertheless, the published contributions have reported more than two hundred organic compounds present in the juice and belonging to a variety of chemical classes (NAGY et al., 1977; PINO & TORRICELLA, 1983), many of which contribute to fresh orange flavour (SHAW et al., 1977), while others cause off-flavours (TATUM et al., 1975).

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This paper reports on experiments aimed toward extending knowledge of the composition of the volatile flavour components in recovered condensates obtained in orange juice concentration. This material is a good source for studies directed toward the characterization of the flavour of fresh orange juice.

1. Materials and methods

1.1. Materials

Recovered orange condensate was obtained directly from the first stage of a juice evaporator used in producing concentrated juice from Valencia oranges (*Citrus sinensis* L. Osbeck) in a local factory in Matanzas, Cuba.

Reagents used were of analytical grade (British Drug House, England) and solvents were redistilled on a fractionating column.

1.2. Extraction methods

Several batches of recovered condensate (1.5 kg each time) were extracted with three portions of 100 cm^3 of redistilled diethyl ether (PINO et al., 1977). The combined extracts were dried over anhydrous sodium sulphate and concentrated to a small volume at 38 °C using a fractionating column.

1.3. Gas-chromatographic analyses

The samples were chromatographed on a Packard—Becker 419, a combined GC-MS Finnigan 3000 D or a Pye Unicam 105, all equipped with flame ionization detectors.

The Packard—Becker 419 was equipped with 3 m × 4 mm i.d. glass columns packed with PEGA, 5% w/w on 60/80 mesh Chromosorb G or SE-30, 15% w/w on 60 per 80 mesh Supasorb. The column oven temperature was programmed at 5 °C min^{-1} from 80 °C to 220 °C. Argon was used as the carrier gas at 25 cm^3 min^{-1} and the temperature of the injector and detector was 220 °C. The peak area measurements were made by an Autolab 6300 digital integrator.

The Finnigan 3000 D was equipped with a 80 m × 0.25 mm i.d. capillary glass column coated with PEG 20 M. The column oven temperature was programmed at 5 °C min^{-1} from 70 °C to 220 °C. Helium was used as the carrier gas.

A glass column 4.6 m × 9 mm i.d. packed with PEG 20 M, 15% w/w on 60/80 mesh Diatomite C was fitted to the Pye Unicam 105. Elution fractions from collection runs were condensed in glass U-tubes immersed in an ice bath.

1.4. Column-chromatographic and thin-layer-chromatographic analyses

In the column-chromatographic technique it was preferred to obtain only two defined fractions, hydrocarbons and oxygenated compounds, in place of many fractions as reported by other authors (SCHREIER et al., 1977), with a view to pre-fractionate the sample and at the same time, to evaluate the weight ratio of hydrocarbons and oxygenated compounds.

The aroma extract (33 g) was separated on 70/230 mesh silica gel 60 (activity 2–3) in a weight ratio 1 : 20. The column was eluted with pentane until no more hydrocarbons were being eluted and the oxygenated compounds were eluted with diethyl ether. Solvents were removed from each fraction in a rotary evaporator (room temperature at 133 kPa). The hydrocarbon fraction was distilled on a fractionating column under vacuum to separate the residue consisting mainly of sesquiterpene hydrocarbons.

The thin-layer-chromatographic analyses of the 2,4-dinitrophenylhydrazones of the carbonyls were carried out using the chromatographic systems reported earlier (PINO, 1980). The thin-layer chromatographic analyses of isolated esters were made on silica gel G plates activated at 110 °C during 4 hours and eluted with cyclohexane–diethyl ether (1 : 2 v/v) or benzene. The spots were revealed with 2% vanillin in concentrated sulphuric acid.

1.5. Chemical separation methods

The α,β-unsaturated aldehydes were isolated from 12 g of aroma extract by reaction with a solution of Na_2SO_3 (1.7 g) $NaHCO_3$ (1.2 g) and 15 cm^3 of distilled water, during 8 hours. The α,β-unsaturated aldehydes were regenerated from the aqueous solution by addition of 10% NaOH solution and extracted with diethyl ether. The aroma extract free of unsaturated aldehydes was mixed with a solution of 38% $NaHSO_3$ to isolate aliphatic aldehydes and methyl ketones. These carbonyl compounds were regenerated in a similar manner to the unsaturated aldehydes. The aroma extract free of the main carbonyl compounds was partitioned with propylene glycol and carbon tetrachloride to isolate the alcohols as reported by HUNTER and MOSHONAS (1965). The esters were isolated from the aroma extract free of carbonyl compounds and alcohols by a column-chromatographic technique eluting the non-polar compounds with pentane and the esters with diethyl ether. The ester fraction was concentrated by distillation under reduced pressure (133 kPa).

The volatile acids were isolated from 6000 cm^3 of recovered condensate with a technique reported by ATTAWAY and co-workers (1964), while the volatile amines were also isolated from 6000 cm^3 of recovered condensate by a technique reported by PINO (1981).

Table 1

Summary of identified volatile compounds in recovered condensate obtained in orange juice concentration

Compounds	Method of identification[a]
Hydrocarbons	
α-thujene	MS, GC
α-pinene	MS, IR, GC
sabinene	MS, IR, GC
Δ^3-carene	MS, GC
myrcene[b]	MS, IR, GC
α-terpinene	MS, GC
limonene[b]	MS, IR, GC
β-phellandrene	MS, GC
γ-terpinene	MS, GC
p-cymene	MS, GC
terpinolene	MS, GC
α-copaene	GC
β-copaene	GC
β-caryophyllene	IR, GC
α-humulene	IR, GC
valencene	IR, GC
Aldehydes and ketones	
acetaldehyde[b]	UV, GC, TLC
acetone	MS, UV, GC, TLC
n-butanal	UV, GC, TLC
butanone	UV, GC, TLC
n-pentanal	GC
n-hexanal	UV, GC, TLC
n-octanal	UV, GC, TLC
n-nonanal	UV, GC, TLC
n-decanal	MS, UV, GC, TLC
n-undecanal[b]	UV, GC, TLC
n-dodecanal	MS, UV, GC, TLC
n-tetradecanal[b c]	UV, GC, TLC
neral	MS, IR, GC, TLC
geranial	MS, IR, GC, TLC
carvone	MS, IR, GC
furfural	UV, GC, TLC
diacetyl	UV, TLC
Alcohols	
methanol	MS, GC
ethanol[b]	MS, GC
2-methyl-1-butanol	MS, GC
3-methyl-1-butanol[b]	MS, IR, GC
1-pentanol	GC
2-hexanol[c]	MS, GC
cis 3-hexen-1-ol	MS, GC
1-heptanol	GC
1-octanol	SC
cis 2,8-*p*-menthadien-1-ol	MS, GC
linalool	MS, IR, GC
terpinen-4-ol	MS, IR, GC
α-terpineol[b]	MS, IR, GC
β-terpineol[c]	MS, GC
nerol	MS, IR, GC
citronellol	MS, IR, GC
geraniol	MS, IR, GC
cis carveol	MS, GC

(Table 1 continued)

Compounds	Method of identification[a]
Esters	
ethyl acetate	MS, GC
linalyl acetate[b]	MS, GC, TLC
terpinyl acetate	MS, GC, TLC
neryl acetate[b]	MS, GC, TLC
geranyl acetate[b]	MS, GC, TLC
methyl butyrate	MS, GC
ethyl butyrate[b]	MS, IR, GC
octyl butyrate	MS, GC
Acids	
acetic	GC
butanoic	GC
hexanoic	GC
heptanoic[c]	GC
octanoic[b]	GC
nonanoic[c]	GC
decanoic	GC
undecanoic[c]	GC
dodecanoic[c]	GC
tetradecanoic[c]	GC
hexadecanoic[c]	GC
Amines	
ethyl anthranilate[b]	MS, IR, NMR

[a] MS, IR, NMR, UV, GC, TLC identification by mass, infrared, nuclear magnetic resonance and ultraviolet spectras, gas chromatography and thin-layer chromatography, respectively

[b] Major components in the family group class

[c] Newly found orange volatile component

1.6. Spectral methods and comparison samples

Infrared spectra of liquid film samples were obtained on a Carl Zeiss UR-20 spectrophotometer, mass spectra on a Hitachi RMU-6 D spectrometer with electron impact source, nuclear magnetic resonance spectra on a Hitachi H-60 spectrometer and ultraviolet spectra on a Pye Unicam SP-800 spectrophotometer. Authentic samples of compounds were purchased from commercial sources to compare with the isolated compounds.

2. Results

Table 1 lists the compounds identified by this combined study with the means of identification. The concentrations of a large number of the components separated from the complex mixture are too low for practical routine analyses and therefore, they could not be identified and other methods will be required. Although we identified 71 compounds, compared to 205 known

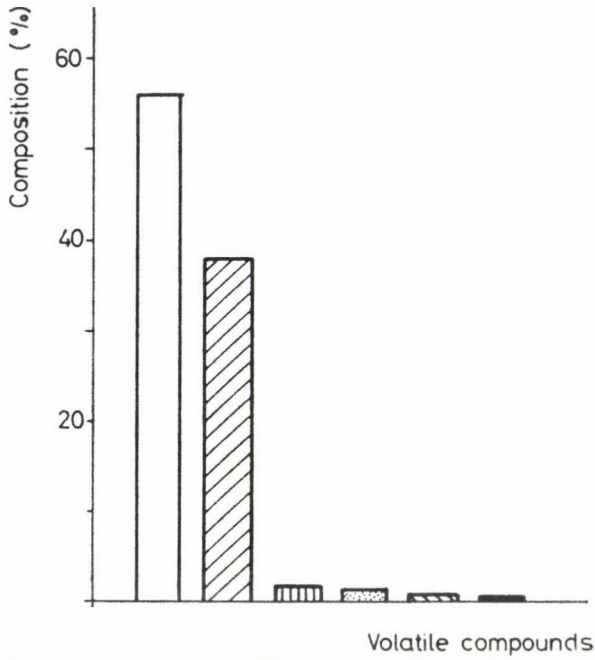

Fig. 1. Quantitative distribution of different types of volatile compounds in recovered condensate obtained in orange juice concentration. ▭: terpenic hydrocarbons (56.0%); ▨: alcohols (38.0%); ▥: aldehydes and ketones (1.7%); ▦: esters (1.4%); ▧: acids (0.6%); ■: amines (0.4%)

orange juice volatile components, no single study listed a majority of the 205 components (PINO & TORRICELLA, 1983).

In the isolated acid fraction, the newly found acids are not strongly identified and therefore they are reported tentatively. The only identified amine was reported earlier by PINO (1981).

From the standpoint of flavour, the actual weight ratios and the threshold values of the various functional group classes, particularly the oxygenated compounds, are very important. The proportions of hydrocarbons, aldehydes and ketones, esters, alcohols, amines and volatile acids are shown in Fig. 1. Of the components listed, the most predominant are the hydrocarbons and alcohols with lower quantities of the other components. The isolated compounds account for about 98% of the total composition in the orange aroma from recovered condensate.

3. Conclusions

The techniques used in this systematic analysis of volatile components of recovered condensate obtained in Valencia orange juice concentration fulfilled the requirements for development of more routine procedures. Using

these techniques 71 components were isolated and identified. Three of them that had not previously been reported as orange volatiles are: 2-hexanol, β-terpineol and *n*-tetradecanal, while C_7 to C_9, C_{11}, C_{12}, C_{14} and C_{16} carboxylic acids are tentatively also reported for the first time.

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EFFECT OF CHRONICALLY ADMINISTERED LARGE AMOUNT OF "SWEET" PAPRIKA ON SOME MUSCLE CHARACTERISTICS

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Young (4-month old) and aged (24-month old) Sprague Dawley rats were kept for an extended period on a semisynthetic diet containing 35% ground, capsaicin free red paprika, while in the control group the paprika was substituted by alfalfa roughage. After an adequate period the animals were killed and the rectus femoris as well as the soleus muscles (representatives of fast twitch and slow twitch muscles, respectively) were quickly excised and immediately cooled by liquid N_2. In addition the left ventricle was also sampled. From the skeletal muscle sarcoplasmic reticulum (SR) and myosin was prepared by conventional methods. From the SR the Ca^{2+}-uptake and the myosin were used for Ca^{2+} as well as K^+-EDTA activated adenosine triphosphatase (ATPase) determinations.

The SR of aged control rat skeletal muscle showed a significantly higher Ca^{2+}-uptake compared to SR prepared from muscle of young rats, however, there was no difference when the aged rats were on paprika diet.

The myosin ATPase activity of aged rat skeletal muscle was also significantly higher as compared to the results obtained from young rats. In the experimental group receiving high amount of paprika for an extended period the myosin ATPase activities were the same as found in young rats.

The myosin ATPase activities of the left ventricles in aged rats on paprika diet were higher than that of young control rats, while in the control aged rats the myosin ATPase activities decreased in comparison to the values in young animals.

On examination by the naked eye the muscles of paprika fed rats were similar to those of young rats, while in the control aged rats the skeletal muscle showed signs of muscle atrophy, degeneration and dedifferentiation.

Keywords: paprika feed, muscle characteristics of rats, ATPase activities, senescence

Red paprika is a well known conventional spice used worldwide in the food industry as well as in the household. In the food industry it is used mainly as food colourant and spice, but it is well known that it contains several biologically active substances (e.g. vitamin C, carotenoids, etc.). Capsaicin is responsible for the pungent characteristics of red paprika and several papers were published on the physiological effect of capsaicin in ground paprika (JANCSÓ, 1955; SAMBAIAH & SATYANARAYANA, 1982; CHAHL et al., 1985). There are, however, few reports on the biological-nutritional effect of capsaicin free paprika either in animals or in humans (Hőgyes, 1878 and others up to 1934 cited in OBERMAYER, 1935; MITKOV, 1968). Recently some experiments

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were reported concerning the effect of ground paprika in the diet of chicken after long term administration, e.g. effect on the colour of egg yolk, etc. (JAKABFI, 1971; HÄRTEL, 1971).

Commercially many varieties of paprika are available and the capsaicin free paprika is called "sweet" paprika. Although it is used widely as a spice and colouring substance, this is one of the first reports concerning its physiological effect as a replacement in the diet. One interesting finding is that large amounts (e.g. 20–35 per cent of the total daily intake) of paprika showed no pathological side-effects (BARNA, 1973a).

The present findings indicate that long-term feeding of large quantities of paprika moved some biochemical characteristics of the aged muscles in the direction we usually find in young animals.

1. Materials and methods

1.1. Experimental animals and their housing

Sprague Dawley CFY male rats from the LATI (Laboratory Animals Institute, Gödöllő, Hungary) were used in these experiments, 6 on paprika diet and 5 young and 5 aged rats on paprika free semisynthetic diet for control purposes. The aged animals were killed at the age of 24 months, while the young males from the same strain were killed at the same time. The animals were housed in normal environment (20–23 °C, 60–70% relative humidity and 10 h per day artificial light).

1.2. Diets and feeding regime

The animals had free access to water and food. A special self-feeder was constructed (BARNA, 1973b) to avoid loss and to register the food intake. The semisynthetic diet contained commercially available (Canned Food and Paprika Industrial Co., Kalocsa) capsaicin free ground paprika up to 35% of the dry weight. In the control group the paprika was replaced by alfalfa roughage. The daily diet as well as the chemical composition are shown in Table 1.

The feeding period lasted 21 months. All cages were supplied with wood sticks to ensure normal tooth wearing.

1.3. Sampling and biochemical procedures

At the end of the experiments the animals were killed by decapitation, exsanguinated and the following muscles quickly excised: rectus femoris (outer layers) and soleus as representatives of fast-twitch (white) and slow-twitch (red) muscles, respectively, and the heart. The latter was further dissected and only the free wall of the left ventricle used. The samples were labelled and immediately frozen and transported in liquid N_2 for biochemical

Table 1

Composition of the diets

Components	Semisynthetic diet	
	spice free	containing paprika
	(%)	(%)
Caseine	7.0	7.0
Milk powder	18.5	18.5
Wheat flour	44.0	17.0
Starch	21.0	21.0
Alfalfa roughage	5.0	—
Paprika powder	—	32.0
Bran	0.74	0.74
Vitamin premix (Phylaxia)	0.23	0.23
Mineral premix (Phylaxia)	3.03	3.03
NaCl	0.50	0.50
Chemical analysis		
Dry matter	91.27	90.13
Crude protein	16.13	17.18
Crude fat	5.57	8.91
Crude fiber	1.39	7.05
N free extract	60.89	49.56
Ash	7.29	7.43

studies to the laboratories of the Boston Biomedical Research Institute. Accumulation of lipofuscin in samples was proved by histopathological examination.

Regarding the small quantities of individual samples for myosin or SR preparation, three by three were pooled then prepared and biochemically analysed.

Calcium uptake by the sarcoplasmic reticulum was determined by the use of cryostat sections as described earlier (MABUCHI & SRETER, 1978). This method enabled us to determine calcium uptake ability of the sarcoplasmic reticulum in small samples and gave good reproducibility. Protein was determined by a micro-biuret method (ITZAKI & GILL, 1964).

Myosin was prepared from the skeletal muscles as well as from the left ventricles by a method described earlier (SRETER et al., 1975). After protein determination, the Ca^{2+}-activated and the K^+-EDTA activated myosin ATPase was determined in a medium containing 50 mmol tris HCl, pH 7.6; 0.025 mol KCl; 10 mmol $CaCl_2$; 1 mmol ATP for Ca^{2+} activation and 50 mmol tris HCl, pH 7.6; 0.6 mol KCl, 5 mmol EDTA and 1 mmol ATP for K^+-EDTA activation. After 1 min incubation in a 25 °C water bath the reaction was stopped by ice-cold trichloroacetic acid (5% final concentration) and by cooling in an ice-bucket. The liberated phosphate was determined by spectrophotometry as described earlier (SRETER et al., 1975).

2. Results

Figures 1 and 2 illustrate the myosin ATPase activities in presence of Ca^{2+} (Fig. 1) and K^{+}-EDTA (Fig. 2).

In skeletal muscles containing either fast-twitch type fibers (rectus femoris) or slow-twitch type fibers (soleus) ageing showed an increased Ca^{2+}-activated myosin ATPase activity, while in the left ventricle this value was somewhat lower than in the control young animals. The effect of long term paprika feeding significantly changed this pattern: in all skeletal muscles the values were similar to those found in young animals. It is interesting that in myosin prepared from the left ventricle of aged controls the Ca^{2+}-activated myosin ATPase activity was lower than in the young animals. Paprika-feeding changed this pattern and gave an even higher ATPase activity as found in the young group. These changes are less evident if K^{+}-EDTA was used to activate myosin ATPase.

Figure 3 shows the results of Ca^{2+}-uptake by sarcoplasmic reticulum vesicles. It is interesting that in aged animals the Ca^{2+} uptake was in both types of muscles significantly higher than in the normal young animals. This was especially marked in the soleus muscles. It should be noted that the Ca^{2+}-uptake in the SR of fast-twitch muscles is significantly higher than in SR of slow-twitch muscles (SRETER, 1969), due to functional differences between these two types of muscles. In the experimental group the feeding of paprika reversed the effect of ageing: the Ca-uptake was similar to that in young animals.

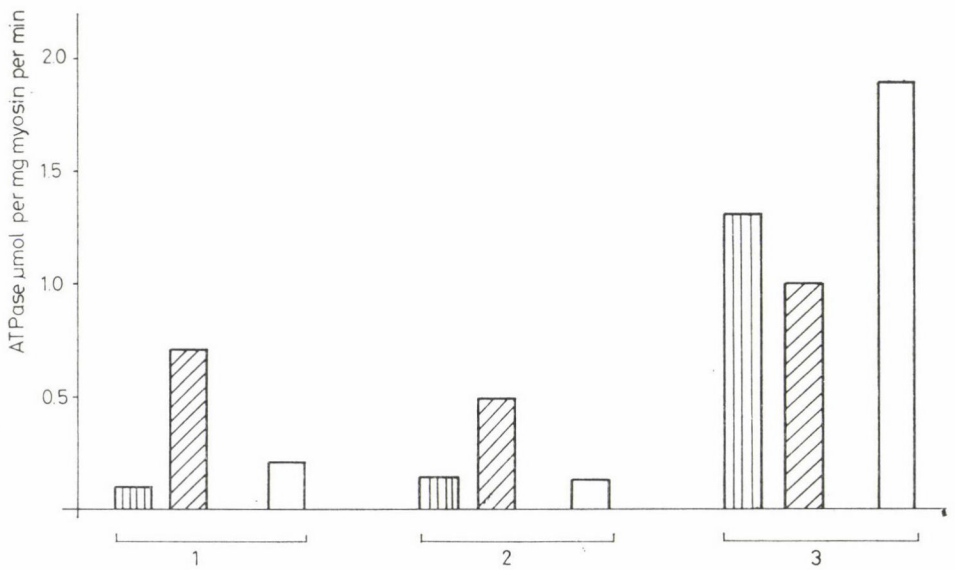

Fig. 1. Ca^{2+} activated ATPase activity of myosin. 1: m. rectus femoris, 2: m. soleus, 3: cardia; ▥: young control rats, ▨: aged control rats, □: aged rats on paprika diet

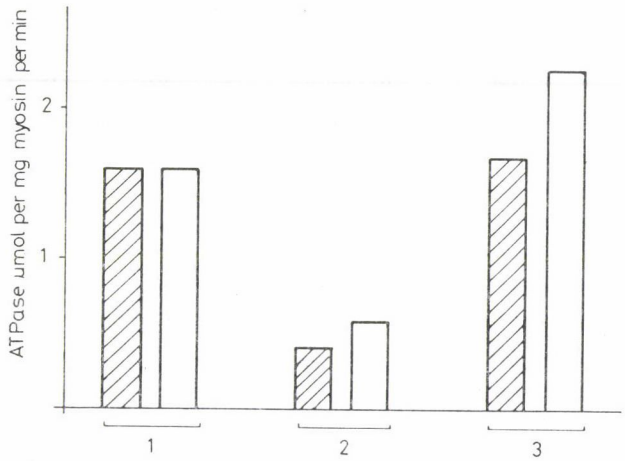

Fig. 2. K^+-EDTA activated ATPase activity of myosin. For legends see Fig. 1

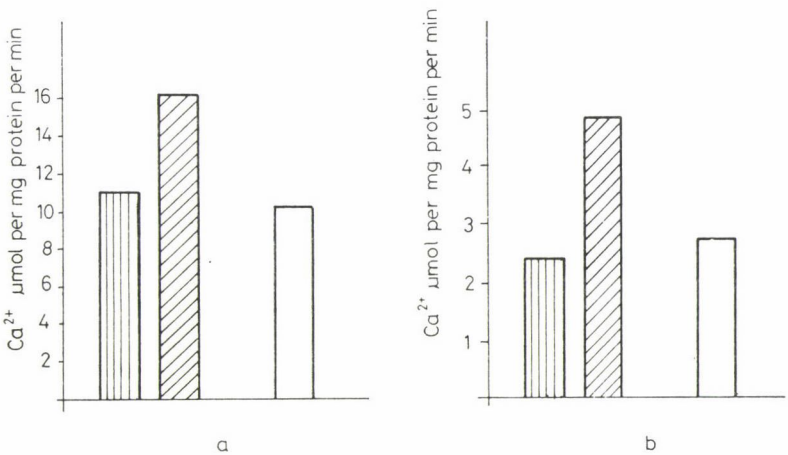

Fig. 3. Ca^{2+}-uptake activities of sarcoplasmic reticulum. For legends see Fig. 1; a: fast-twitch muscle, b: slow-twitch muscle

3. Discussion

It is well accepted that there are two major skeletal muscle fiber types: slow-twitch fibers and fast-twitch fibers. Most of the skeletal muscles, however, are mixed, containing both types of fibers, but according to functional demands certain muscles contain mostly fast-twitch type fibers (e.g. the outer layers of the rectus femoris muscle) or slow-twitch type fibers (e.g. soleus). The immediate energy source for contraction is supplied by the breakdown of ATP, which is regenerated by oxidative phosphorylation. Myosin, the most

important muscle protein plays a vital role in the contraction cycle by attaching to the actin filaments and has a unique ability to split ATP in order to supply the energy needed. The physiological characteristics of the two types of muscle fibers are markedly different. Muscle fibers of the fast-twitch type are normally activated by brief bursts of high frequency impulse activity while the slow-twitch type muscles are involved in long lasting tonic contractions needed, e.g. in posture. Heart muscle is a special type mixed muscle and the structure and function of its myosin molecule is somewhat similar to the slow-twitch type myosin (e.g. similar heavy and light chain; lower ATPase activity, similar electrophoretic pattern on polyacrylamide gels, etc.). Heart muscles, however, contain less developed SR system and it is believed that not only the SR but mitochondria also plays an important role in the Ca-uptake and release mechanism in the heart.

The present results indicate a definite shift in the ATPase activities of aged animals, and the feeding of paprika was able to reverse this phenomenon.

Fragmented sarcoplasmic reticulum preparations from aged fast-twitch or slow-twitch muscles consistently picked up significantly more calcium than their young counterparts. Feeding of paprika to old animals completely reversed this phenomenon and as we demonstrated, the calcium uptake of SR in aged animals was approximately the same as in young ones. This trend is also true in case of the myosin ATPase activities of the skeletal muscles. In myosin extracted from the cardiac ventricle the decreased Ca-activated ATPase activity observed in aged animals was reversed in paprika-fed animals and was significantly higher than that we found in the young animals.

The present results – as a preliminary informative observation – suggest that feeding of large amount of paprika on a systematic basis is advantageous since the muscle composition and function does not show the sign of ageing. It is likely that its biological effect is a retardation of the ageing process in the skeletal muscles.

Future research designed on large number of animals is needed for the identification of the bioactive components of paprika responsible for the effect on muscles, for the determination of the paprika level in the diet and duration of feeding period which are effective and finally for the determination of the physiological consequences of the biochemical changes caused by the paprika in muscles.

*

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EVIDENCE FOR THE PRESENCE OF LIPOXYGENASE AND HYDROPEROXIDE-DECOMPOSING ENZYME IN RED PEPPER SEEDS

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Lipoxygenase (ECI.13.II.12) was investigated for its existence and biochemical properties in fresh and dry red pepper seeds. The enzyme was found to be of low concentration and requires continuous oxygen supply. The change in optical density at 234 nm was used to determine the enzyme activity. During the reaction periods, an increase followed by a decrease in absorbance has been observed indicating the presence of hydroperoxide decomposing activity in the crude extract. The reaction rate and products of these enzymatic systems were investigated with an automatic spectrophotometer. Three peaks of absorbance appeared at 212, 225 and 234 nm during the reaction periods up to 40 minutes.

Lipoxygenase activity completely disappeared when EDTA and ascorbic acid were added to the extracting media but no effect of these compounds was observed on hydroperoxide decomposing activity.

It was found that the crude extract of dry seeds has an inhibitory effect on soybean lipoxygenase revealing that there is either inhibitor or antioxidant in the seeds of red pepper.

Keywords: lipoxygenase, hydroperoxide, enzyme activity, red pepper seeds.

Red pepper processing technology uses the skin of the fruits to obtain high intensity red colourant. Other products, like seeds are by-products. Up to 10%, seeds are allowed in the ground product owing to the antioxidant and colour-stabilizing action of the seeds. Nevertheless, substantial discolouration takes place during the storage of the ground red pepper. Lipid content and lipolytic activity of these seeds may interfere and take part in the product discolouration.

According to SOMOS (1981) the different cultivars of red pepper contain 0.4% lipids, and the most common fatty acids in red pepper oil are oleic, linoleic and palmitic acids. Like any vegetable, red pepper oil would be attacked by many oxidative enzymes leading to undesirable off-flavour and discolouration during storage and processing.

PINSKY and co-workers (1971) investigated the activity of lipoxygenase (LOX) in many fruit and vegetable tissues including red pepper. He found that LOX activity in red pepper was very low in comparison to the activity of LOX in other vegetables like egg plant and cauliflower.

It was found that LOX activity in some seeds is accompanied by hydroperoxide decomposing enzymes which produce α-keto isomers of linoleic acid

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hydroperoxide or other degraded products. These enzymes cause a decrease in the absorbance at 234 nm during analysis by spectrophotometry. Hydroperoxide isomerase was first found in flax seeds in 1966 as mentioned by ZIMMERMAN and VICK (1970) then in some plant tissues (ZIMMERMAN et al., 1974) and in some cereals (GRAVELAND, 1973).

Because of the low lipolytic enzyme content of some plant products, solubility problems and the interaction between their activities, the method of assay faces some difficulties. On the other hand, the enzyme systems, nature and reaction product of hydroperoxide decomposing enzymes has not been sufficiently investigated to give a clear view on their role in lipid oxidation and other biological effects.

Our aim was to establish whether oxidative enzymes like LOX and hydroperoxide decomposing activity were found in the seeds of red pepper.

1. Materials and methods

1.1. Materials

Fresh red pepper and dry seeds were obtained from the Paprika Feldolgozó Vállalat, Szeged (Paprika Processing Co., Szeged). Pure free linoleic acid, Tween 20 and soybean LOX were purchased from Sigma (USA). Redistilled water was used in the preparation of the different solutions.

1.2. Substrate preparation

Linoleic acid substrate was prepared according to the method of SEKHAR and REDDY (1982). Half cm^3 linoleic acid, 0.5 cm^3 Tween 20 and 40 cm^3 of 0.1 mol borate buffer (pH 7) were sonicated in a Tesla ultrasonic device (Czechoslovakia) for 5 min and used as the stock solution, which should be stored in a refrigerator when not in use, and then diluted to 200 cm^3 with borate buffer.

1.3. Crude enzyme preparation

The method of ABBAS and co-workers (1979) was used to prepare the enzyme extract. Ten of fresh seeds were diced and macerated in a mortar with a pistle in a small volume of 0.05 mol phosphate-borate buffer, pH 7, containing 0.5% v/v Triton X-100, then the volume was completed to 100 cm^3 with the same buffer. The macerate was shaken for 30 min in a mechanical shaker and then strained through a double layer cheese cloth and centrifuged at 20 000 r.p.m. for 20 min at 4 °C in a Beckman (USA) type JA-20 refrigerated centrifuge. The supernatant was taken and used as crude extract. Dry seeds were milled in a coffee mill to about 20 mesh powder, then extracted with the same method with or without Triton X-100.

1.4. Enzyme assay

Two methods were applied to measure LOX activity. The first one was that of SEKHAR and REDDY (1982) which consists of extraction of reaction product with absolute alcohol and determination of increase in optical density at 234 nm. One cm^3 of enzyme extract was taken in a test tube and 8 cm^3 of diluted substrate at the desired pH was added to it. Oxygen was passed continuously into the reaction mixture during the experiment. From this reaction mixture 1 cm^3 aliquot was transferred to a test tube containing 2 cm^3 of absolute alcohol, and 7 cm^3 of 60% alcohol was added to make a total volume of 10 cm^3. Half cm^3 of this mixture was taken into a quartz cuvette and diluted to 3 cm^3 with redistilled water to read the optical density at 234 nm in the automatic spectrophotometer (Spectrod M-40, Zeiss, Jena, GDR).

The second method was that of AL-OBAIDY and SIDDIQI (1981). The method involved initiation of the reaction in the Spectrophotometric cell containing 0.2 cm^3 of Tween-20 solubilized substrate at the desired pH; 0.1 cm^3 of enzyme extract and 3 cm^3 of redistilled water. The increase in absorbance was read at 234 nm.

Hydroperoxide decomposing activity was assayed according to the method used by ZIMMERMAN and VICK (1970) with some modification. Linoleic acid substrate prepared for LOX assay was autoxidized to hydroperoxide by bubbling oxygen through it and keeping it overnight at room temperature. Amounts of 0.2 cm^3 of this substrate, 3 cm^3 redistilled water and 0.1 cm^3 of crude enzyme extract were mixed well in the spectrophotometer cuvette. The decrease in absorbance at 234 nm was used to determine the activity of hydroperoxide decomposing enzyme. The blank was prepared by the same method but with previously heat inactivated enzyme extract.

The Spectrod M-40 was used to investigate the changes taking place in the reaction mixture by automatically measuring the spectra of reaction products between 200–909 nm.

All experiments were done in duplicate and the values in the results represent the mean of at least 2–3 measurements.

2. Results and discussion

2.1. Effect of time on enzyme activity

With the method of AL-OBAIDY and SIDDIQI (1981) in which the enzyme extract volume was small, the crude extract showed no measurable LOX activity. It was thought that the enzyme either existed in low concentration in the seeds and needed longer time to give a measurable effect on its substrate or that it was strongly bound to the cell membranes or other seed components. In order to see the effect of long reaction times and high enzyme

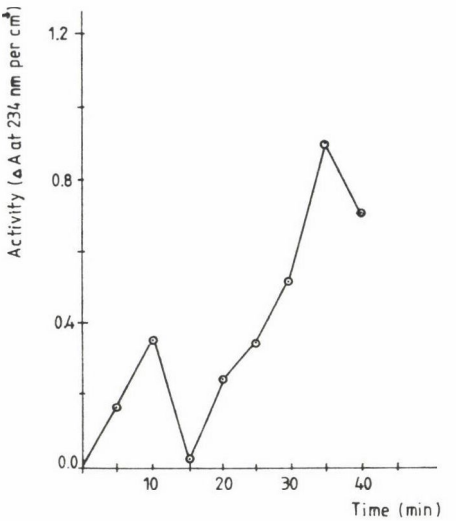


Fig. 1. Effect of reaction time on the activity of lipoxygenase from fresh red pepper seeds

concentration, the method of SEKHAR and REDDY (1982) was used to measure enzyme activity. Moreover, the enzyme was extracted with phosphate buffer containing 0.5% Triton X-100 as solubilizing agent using the procedure of FEYES and co-workers (1982). The results of this experiment are shown in Fig. 1. During 15 min of reaction there was an increase in absorbance at 234 nm indicating the presence of LOX activity. After 15 min there was a rapid decrease in absorbance to the starting level, then the absorbance returned to increase and eventually decreased. This fluctuation in absorbance lead to the assumption that hydroperoxide decomposing agent was present acting simultaneously with lipoxygenase. GARDNER (1975) reviewed the actors causing decomposition of hydroperoxide, he reported these factors as being trace elements or hydroperoxide decomposing enzymes. Accordingly, EDTA was added to the extracting media at 1% concentration. This addition caused complete inhibition of the activity of LOX revealing that EDTA has eliminated some chelating trace elements, like iron, essential for LOX. Hydroperoxide decomposing enzyme activity continued when the enzyme extract was added to autoxidized linoleic acid substrate in the presence of EDTA as shown in Fig. 2. These observations, as well as the absence of any activity in the blank experiment suggested that the observed change in linoleic acid hydroperoxide was due to the action of an enzyme.

The long reaction time required by red pepper seed LOX was similar to that observed with LOX from other sources like tomato and rice in which LOX needed more than 15 min to show activity (BONNET & CROUZET, 1977; SEKHAR & REDDY, 1982).

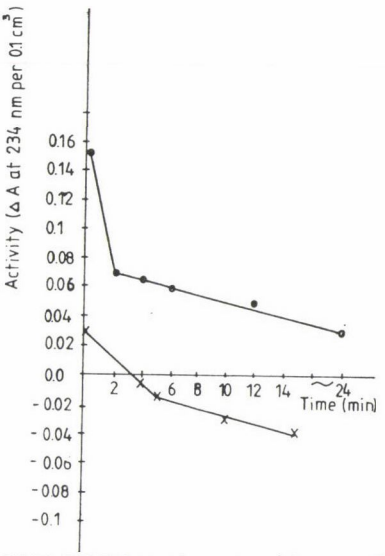


Fig. 2. Effect of addition of 1% EDTA to the extracting media on the activity of lipoxygenase from fresh red pepper seeds. o——o: fresh seeds; ×——×: dry seeds

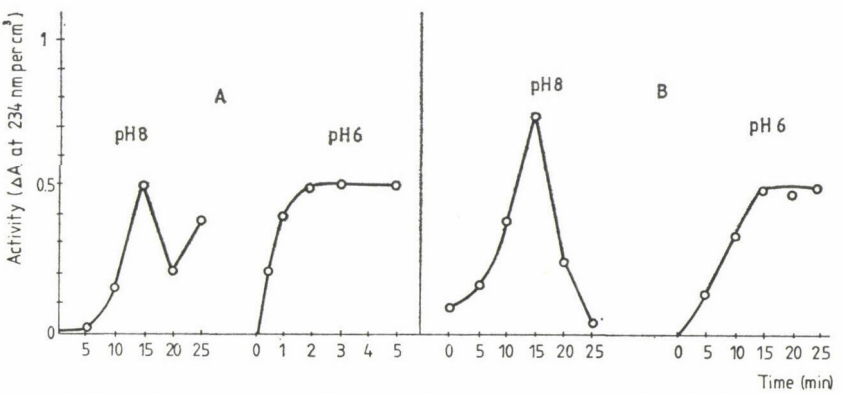


Fig. 3. Activity of lipoxygenase from fresh pepper seeds under different conditions. A: with one drop ethanol in the reaction mixture; B: without ethanol

The extraction obtained without Triton showed no activity, while addition of 0.5% Triton to the extracting buffer solution increased the extractability of the enzyme indicating that LOX is absorbed to cell components or cell membranes.

When the mixture of seed extract and linoleic acid substrate was diluted to 3 cm^3 with water containing one drop of ethyl alcohol and the activity was assayed at two pH values, there was no decrease in absorbance at 234 nm at pH 6, while at pH 8, the decreasing section was longer than without alcohol (Fig. 3). This indicates that LOX prefers acid pH values and alcohol is necessary to increase the solubility of linoleic acid substrate.

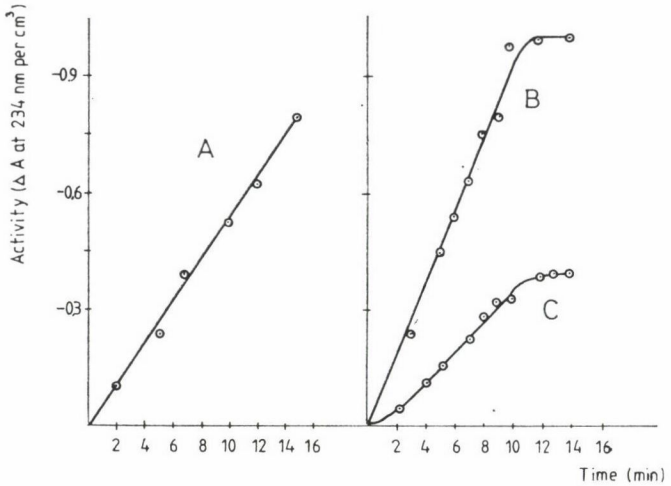


Fig. 4a. Reaction rate curves of hydroperoxide decomposing enzyme of red pepper seeds. A: with 0.1 cm³ substrate; B: with 0.2 cm³ substrate; C: with freshly prepared linoleic acid substrate

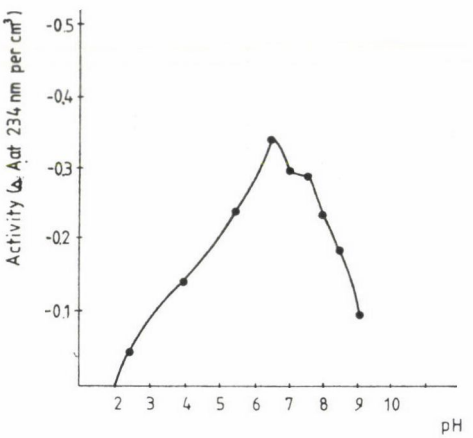


Fig. 4b. Effect of different pH values on the activity of hydroperoxide decomposing enzyme of dry red pepper seeds

All experiments were unsuccessful to extract and measure any LOX activity in dry seeds indicating that irreversible effect had been caused to the enzyme during drying or the enzyme requires more efficient procedure to extract it. This is questionable yet and requires more investigation.

2.2. Hydroperoxide decomposing enzyme in dry seeds

The enzyme was extracted from dry seeds of red pepper with 0.05 mol phosphate–borate buffer (pH 7) with and without Triton. Extracts prepared with Triton showed no activity. This agrees with the study of PINSKY and

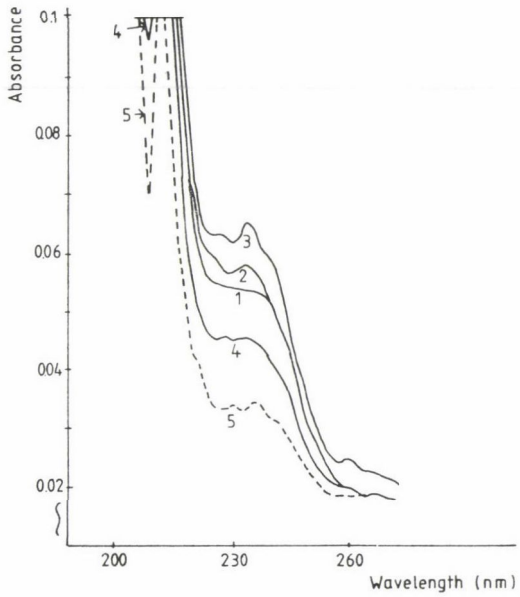


Fig. 5a. Spectra of reaction products of fresh red pepper seed lipoxygenase during time intervals. 1: reaction time 0; 2: 20 min; 3: 30 min; 4: 35 min; 5: 40 min

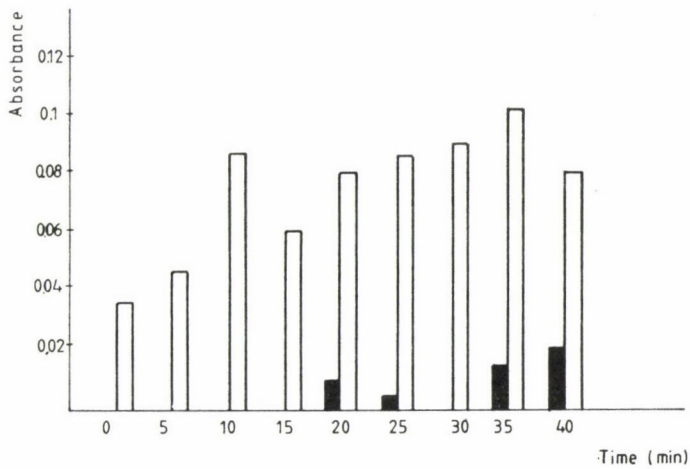


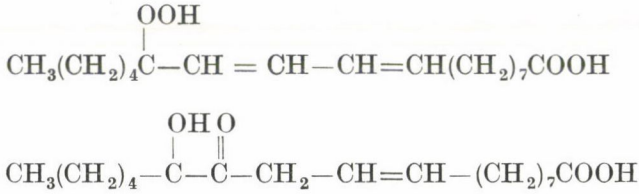
Fig. 5b. Effect of time intervals on the reaction products of fresh red pepper seed lipoxygenase. Data were calculated from the height of two major peaks of reaction product spectra. □: absorbance at 234 nm; ■: absorbance at 225 nm

co-workers (1971) which indicates that Triton is not always useful to extract enzymes from different sources. Extraction without Triton was efficient and reproducible. The reaction rate of hydroperoxide decomposing enzyme is shown in Fig. 4a. Linear relation was obtained when enzyme activity was plotted versus reaction time.

The enzyme showed highest activity at pH between 7 and 8 (Fig. 4b). The study of ZIMMERMAN and VICK (1970) on the enzyme from flax seeds showed that pH 7 was the optimum pH of the activity. Neither dry pepper seed nor flax seed enzymes were affected by EDTA when this was added to the extracting media.

2.3. Spectrophotometric identification of reaction products

The effect of reaction time on the reaction products of fresh red pepper seed extract is shown in Figs. 5a, b. Three peaks were obtained at 234 nm, 225–227 nm and 210–212 nm. The peak at 234 nm refers to linoleic acid hydroperoxide, while the others refer to hydroperoxide decomposition products and they appeared after periods when the decrease in absorbance at 234 nm had taken place. ZIMMERMAN and VICK (1970) found that absorbance maxima between 220–230 nm are characteristic of α-ketols and their derivatives. This supports our assumption that the enzyme found by us in red pepper seeds was linoleic acid hydroperoxide isomerase which causes the isomerization of linoleic acid hydroperoxide to a keto isomer as follows:



CAYREL and co-workers (1983) found two absorbance peaks in their investigation into spectra of grape LOX reaction products, one at 234 nm and the other one at 206 nm without identifying the nature of the latter compound.

The results obtained by calculating the highest extension of each absorbance peak (Fig. 5a) agree completely with those gained by measuring the oxidizing activity of fresh seed extract as shown in Fig. 2.

The effect of hydroperoxide-decomposing enzyme of dry red pepper seeds on the spectra of hydroperoxide was studied by two methods which are shown in Figs 6a, 6b. The decrease in the area under the peak at 234 nm in both of the methods indicates the presence of actual hydroperoxide decomposing enzyme in the crude extract of red pepper seeds.

2.4. Inhibition of soya LOX by red pepper seed extract

Dry seed extract was found to have an inhibitory action on pure soybean LOX. This confirmed the assumption that some natural inhibitors exist in red pepper. PINSKY and co-workers (1971) also found that 0.3 cm^3 of green pepper tissue extract caused 43.3% inhibition to soybean LOX. With red pepper,

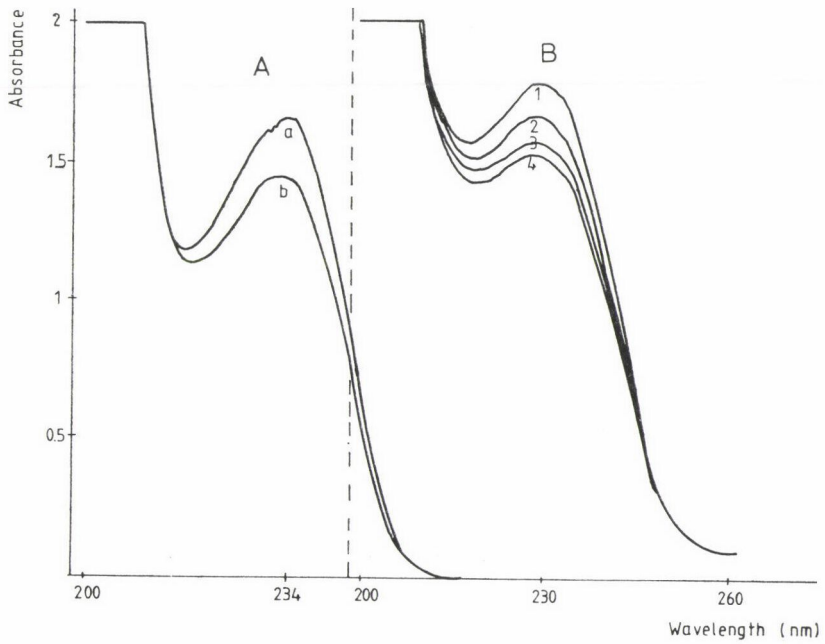


Fig. 6a. Spectra of hydroperoxide decomposition by dry seed extract of red pepper. A: extraction of hydroperoxide by alcohol; B: direct reaction initiation in spectrophotometer; a: blank inactive enzyme; b: active enzyme; 1–4: reaction times (min)

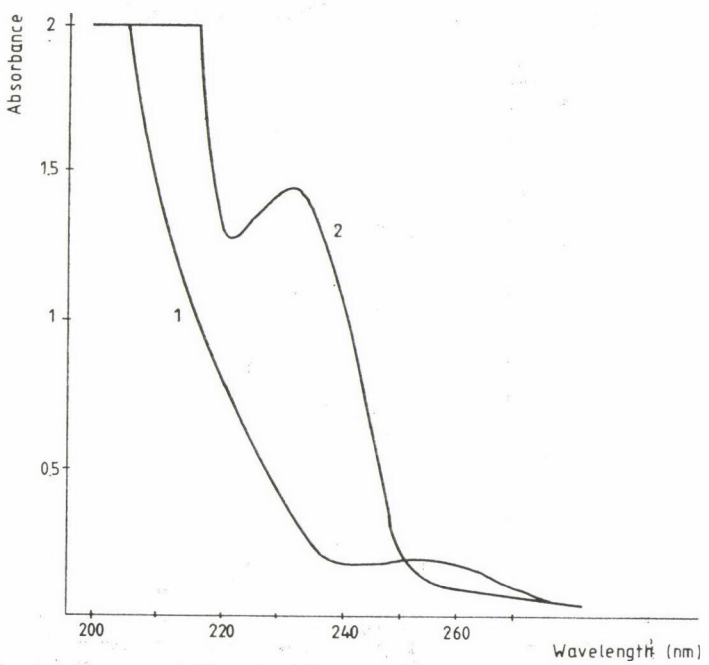


Fig. 6b. Spectra of linoleic acid substrate and crude enzyme extract. 1: crude enzyme; 2: substrate

0.075 cm^3 of dry seed extract caused 58.5% inhibition to soybean LOX at pH 7.5 (Table 1). The reaction rate of soybean LOX decreased proportionally

Table 1

Effect of different concentration of red pepper seed extract on the reaction rate of soybean lipoxygenase

(2 mg of soya LOX were dissolved in 10 cm^3 of phosphate buffer pH 7. Each value represents the mean of three measurements).

Treatments	Reaction rate	Inhibition (%)
Soya LOX (0.05 cm^3) + 0.02 cm^3 substrate	1.34	0
Soya LOX (0.05 cm^3) + 0.05 cm^3 red pepper seed extract	1.00	26.8
Soya LOX (0.025 cm^3) + 0.05 cm^3 red pepper seed extract	0.733	46.5
Soya LOX (0.025 cm^3) + 0.075 cm^3 red pepper seed extract	0.533	61.0
Soya LOX (0.025 cm^3) + 0.075 cm^3 seed extract previously mixed with the substrate	0.566	58.5

Reaction rate was calculated from the slopes of the curves. (Reaction rate = absorbance at 234 nm per min.)

with the increase in the concentration of seed extract of red pepper as shown in Fig. 7, this enabled us to explain when LOX activity is low in fresh seeds and is absent in dry seeds. The presence of many natural lipid oxidation inhibitors and catalysts was reviewed by ERIKSSON (1982).

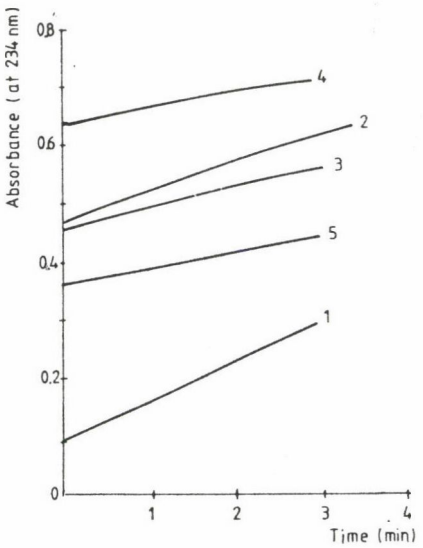


Fig. 7. Reaction rate curves of soybean lipoxygenase in the presence of different concentrations of dry seed extract of red pepper. 1: without seed extract; 2: with 0.05 cm^3 seed extract; 3: with 0.075 cm^3 seed extract; 4: with 0.1 cm^3 seed extract; 5: with 0.1 cm^3 seed extract previously mixed with the substrate

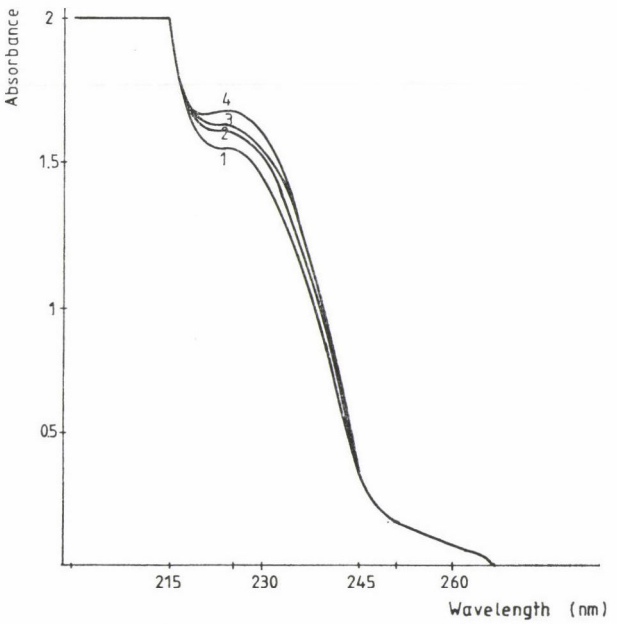


Fig. 8. Spectra of reaction product of soybean lipoxygenase in the presence of 0.15 cm³ of crude dry seed extract. 1–4: reaction times (min)

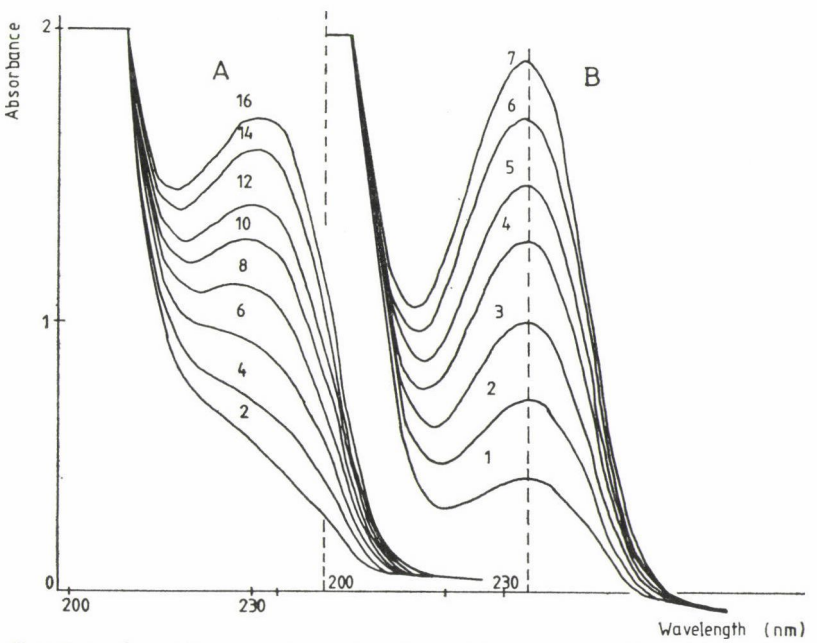


Fig. 9. Spectra of reaction product of soybean lipoxygenase. A: with 0.025 cm³ seed extract, 2–16: reaction times (min); B: without seed extract, 1–7: reaction times (min)

In order to investigate the effect of dry seed extract on the reaction products of soybean LOX, 0.025–0.15 cm^3 of dry seed extracts were added to the mixture of soybean LOX and linoleic acid substrate. The spectra between 200 nm and 909 nm were recorded automatically during 1 and 2 minutes. The results showed an observable change in the form of the peak at 234 nm and new small peaks appeared over 220 nm as shown in Figs. 8 and 9. The change in the form of the peak at 234 nm indicates that an interaction takes place between linoleic acid or its hydroperoxide with some naturally occurring components of dry seeds. Similar observations have been made by PATTEE and co-workers (1982) who reviewed the different possible interactions between lipids and other components in the cells of the legume family. The nature of this interaction was investigated so far by different analytical techniques.

From the spectrum study it was noticed that when soybean LOX activity continued producing more hydroperoxide the peak wave length approached gradually 230 nm, with the appearance of small peaks at 227 nm and 234 nm, but the distance between the peaks was less than when soybean LOX was used alone indicating the inhibiting effect of seed extract. The inhibitory effect may refer to hydroperoxide formation or its degraded products. The inhibitory effect of hydroperoxide and its degraded products was described by MATSUSHITA (1975).

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DETECTION OF FATTY ACID AND FREE FATTY ACID COMPOSITION OF SAFFLOWER SEEDS DURING GERMINATION BY GAS-LIQUID CHROMATOGRAPHY (GLC)

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Safflower seeds are of great importance among other oil seeds, they contain about 38% lipids. The lipids of dry seeds were extracted by two methods. The changes in fatty acid composition of germinated seeds were detected by GLC technique provided with highly efficient integrator. Saturated fatty acids (FAs) and oleic acid concentration decreased during the period of germination while linoleic acid remained unchanged. Analysis of free fatty acids (FFAs) by GLC showed significant increase in saturated FA content of germinated seeds indicating the specificity of safflower seed lipase towards saturated FA esters. Oleic acid concentration increased up to 45 h, then decreased. Linoleic acid decreased within all intervals used in germination due to the biological oxidizing factors which take part in the decomposition of unsaturated FFAs. The relation between lipase activity, oil acidity and water content during germination is also discussed.

Keywords: fatty acid, free fatty acid, safflower seed, gas-liquid chromatography

During storage and technological processes of plant products, like cereals, many changes take place in lipid composition. The biochemical pathways of these changes are initiated and accomplished by certain enzymes. The role of these enzymes in lipid decomposition is shown in Fig. 1.

These enzyme activities are highly stimulated when the seeds are stored under unfavourable conditions (high temperature and moisture) which, in turn, activate the process of germination during long-term storage of many cereals like oil seeds. The results of these processes are the economical loss of lipid yield and deterioration of the organoleptic properties of the final products.

The change in lipid composition during storage of many cereals have been studied. Matsuda and Hirayama (1973) investigated the changes in lipid composition and lipolytic acyl hydrolase activity in rice grain during storage under different conditions. Remarkable changes in lipid components were found during the storage of wheat (Clayton & Morrison, 1972).

The effect of germination on FA composition of many cereal products was the subject of several investigations. Pomeranz and Schellenberger (1961) studied the localization of FFAs during germination of wheat as a

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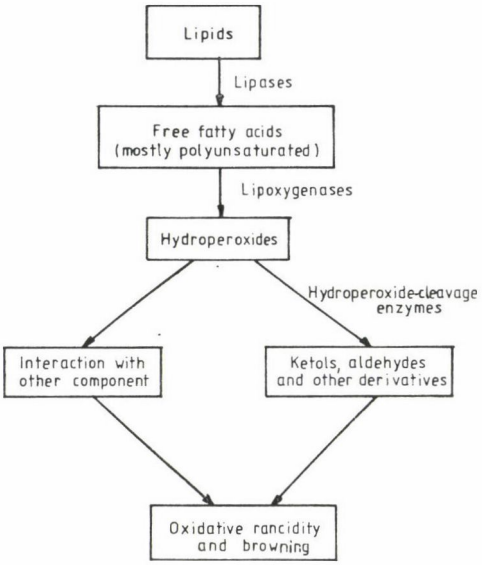

Fig. 1. The scheme of biochemical pathway of changes in lipid composition

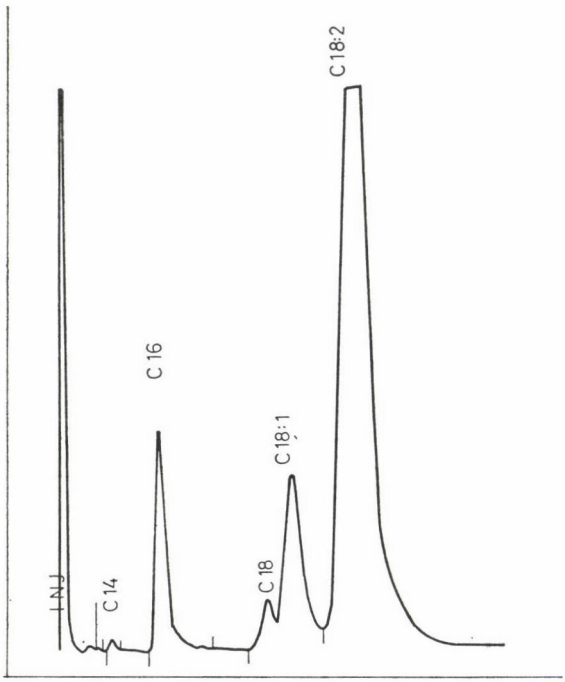

Fig. 2. Fatty acid composition of safflower seed oil by GLC. For conditions, see the text

function of lipase activity, while OCLOTT and FONTAINE (1941) investigated the changes in FFA composition, fat acidity and lipase activity in germinated cotton seeds. The effect on germination of FA composition of peanut treated with growth regulators was studied by VYAS and co-workers (1969). The activity of lipases in relation to lipid hydrolysis of many cereals during germination has been investigated (DRAPRON et al., 1969; NARZISS & SEKIN, 1974; THANKI et al., 1970 and MADYAROV et al., 1975).

Safflower seeds are economically important oil seeds in many countries like India and Iraq. The seeds are rich in essential unsaturated fatty acids as shown in Fig. 2, so the edible oil extracted from this crop is of nutritional and economical importance. The effect of germination on FA composition of safflower seeds was not studied, with the exception that WEISS (1917) reported the changes in the amount of lipid gained from the seeds in the course of germination.

In this work we investigate the changes in FA composition of germinated safflower seeds in relation to lipase activity.

1. Materials and methods

Safflower seeds were obtained from Agronomy Department, College of Agriculture, University of Baghdad. All chemicals used were purchased from BDH (British Drug House, London).

1.1. Seed germination

One hundred and fifty g of dry seeds were germinated at 25 °C in a wide temperature range germinator using moist filter paper towels after sterilization with Clorax. Amounts of 25, 30, 10 and 5 g of seeds were used for lipase activity measurements, lipid extraction for GLC analysis, oil acidity measurements and water content determination, respectively. Samples were taken in duplicate in time periods of 0, 15, 30, 45 and 60 h. The mean of at least two determinations was used in the results with negligible variation.

1.2. Lipid extraction

Two methods were used to extract lipid components of germinated seeds. In Soxhlet method 10 g of seeds were ground in a mortar with pestle and the lipid extracted in the Soxhlet extractor for 16 h using diethyl ether as extracting solvent. The solvent was evaporated in the rotary evaporator under vacuum. The second method was chloroform–methanol (2 : 1) with modified procedure which is shown in Fig. 3. Acidity of extracted oil samples was determined by titration with alkaline solution using α-naphtolphtaline as indicator. Ten cm^3 of 10% oil in ether was titrated with 0.1 *N* alcoholic KOH to the end point.

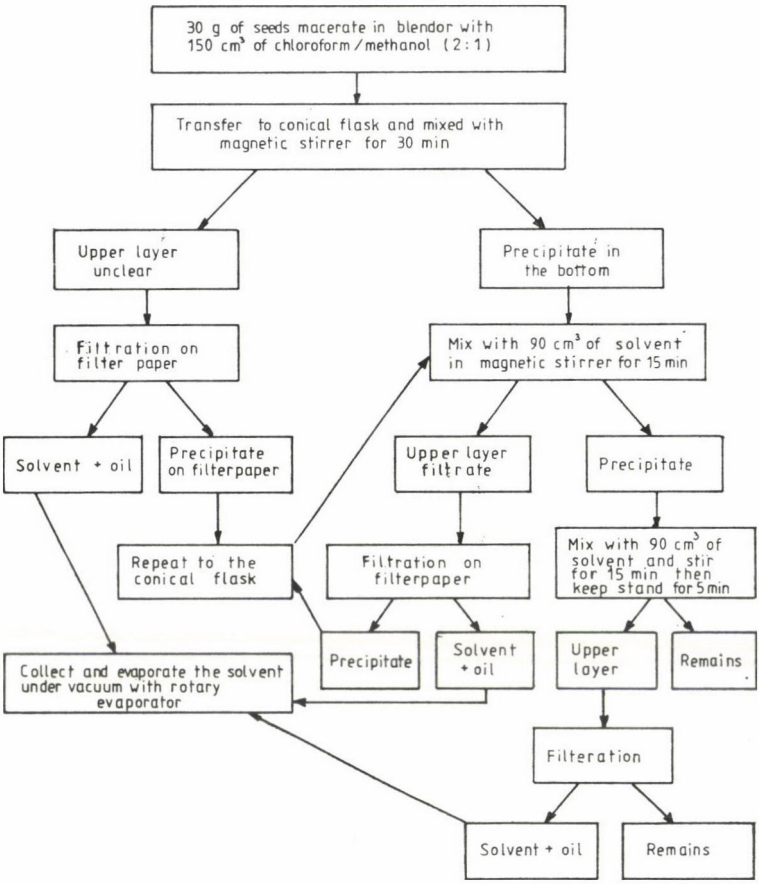

Fig. 3. Scheme for the extraction of lipid compounds of germinated safflower seeds

1.3. Free fatty acid separation

Absorption chromatography was applied to isolate FFAs from other lipid compounds. The column was prepared according to McCarthy and Duthie (1962). Five g of heat-activated silicic acid were added to 10 cm^3 of 4.5% isopropanolic KOH and mixed with 30 cm^3 of dry ethyl ether by continuous stirring, then the mixture was left still for 5 min and transferred to a 20×200 mm glass column containing porous glass disc in the bottom. Ten cm^3 of dissolved oil were carefully applied into the column and eluted with 100 cm^3 pure ether. The column was eluted 4-times with 25 cm^3 of the same solvent. The fractions were collected and the solvent was evaporated in a rotary evaporator under vacuum.

The absorbed FFAs were released by washing the column twice with 50 cm^3 of 2% formic acid in ether. The solvent was then evaporated at 40 °C

under vacuum. Small amount of the solvent should remain to increase the recovery of FFAs. All samples were kept in small vials under nitrogen at −20 °C.

1.4. GLC analysis

Lipid samples of 0.05 g were mixed in small ampoules with 0.2 cm^3 of 0.2 mol sodium methoxide. Esterification was done at 70 °C in an oven for 30 min. For FFA analysis the remained solvent was completely evaporated at 50 °C under nitrogen current. FFAs were then transferred to small ampoules by using ethylene chloride as transferring solvent and esterification was carried out by the same method mentioned above.

GLC analysis was carried out by using 5711a GLC of Hewlett Packard (USA) provided with 330 A integrator. The column was 5% ethylene glycol succinate on Chrom-W 60/80 mesh with a length of 150 cm and 3.17 mm in diameter. The temperatures were 200 °C, 160 °C, and 250 °C for injector, column and detector, respectively. Nitrogen gas flow rate was 24 cm^3 per min, for oxygen 300 cm^3 per min and for hydrogen 24 cm^3 per min. Injection volume was 0.2 μl for FA analysis and 0.5 μl for FFA analysis.

1.5. Assay method for lipase

Titration of released fatty acids with 0.05 *N* sodium hydroxide solution was followed using pH-state method with starting at pH-value of 8 (Bier, 1955). Substrate was triolein emulsified with 5% gum-arabic solution. The "crude enzyme" was prepared with macerating 25 g of seeds in blendor for 5 min, filtrating the macerate through double layer cheese cloth and centrifuging the mixture for 5 min at 0 °C at 10 000 r.p.m.

Five cm^3 of "crude enzyme" was mixed with 10 cm^3 of substrate and incubated for 12 h at 30 °C. Five cm^3 of absolute alcohol was then added to stop the reaction and the released fatty acids were titrated with alkaline solution to pH 8 using Beckman pH-meter (USA).

2. Results and discussion

2.1. Lipid extraction

The results of the two extracting methods which are shown in Table 1 revealed that the chloroform-methanol method was much more efficient than the Soxhlet. This may be due to the ability of the solvent mixture "which contains methanol" to extract FFAs even those strongly bound to the solid components of germinated seeds. Moreover, remaining of very small amount of solvent with the sample increased the recovery of extracted FFAs as it avoided the adhesion of FFA on the flask with some solid materials when complete

removal of solvent is being done. The amount of extracted oil was larger to some extent in Soxhlet method than chloroform-methanol, this could be explained by extracting some water soluble compound by ether, specially, when some moisture exists in the sample.

Table 1

Effect of extraction method on the extractability of lipid and FFAs from safflower seeds

Treatments	0.01 *N* KOH (cm³ per 1 g oil)	
	chloroform–methanol	Soxhlet
Complete removal of solvent	2.12 2.00	1.10 1.00
Not complete removal of solvent	2.80 2.75	2.00 2.05
Lipid (%)	33	36

Lipid (%) was calculated on the basis of dry weight of seeds. The results are the mean values of three determinations

2.2. Effect of germination on lipase activity and oil acidity

During germination periods, the oil acidity and lipase activity increased proportionally with increase of water content in germinated seeds. Increase in water content mostly causes activation to lipase activity which, in turn,

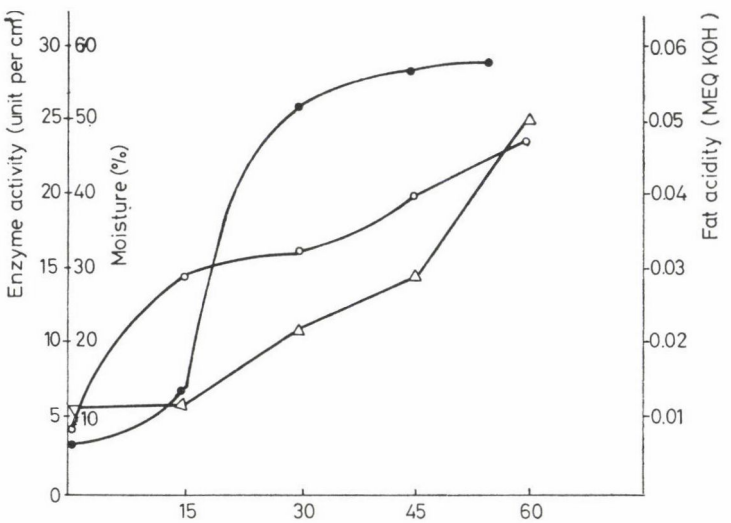

Fig. 4. Effect of germination on fat acidity and lipase activity of safflower seeds. ●—●: enzyme activity; ○——○: % moisture; △——△: fat acidity

hydrolyzes the fatty acid ester of lipid constituents (trigylcerides, phospholipids and glycolipids) resulting FFAs, therefore, the acidity of extracted oil up to 15 h of germination was small since water content of seeds was low. When the water content increased to the maximum after 30 h, the oil acidity increased ascendingly with lipase activity as shown in Fig. 4. OCLOTT and FONTAINE (1941) noticed the same manner of lipase activity and oil acidity in germinated cotton seeds. The increase in lipase activity after 30 h of germination was 10-times more than in ungerminated seeds, this can be explained either by the effects of physiological changes taking place during that stage of germination which might activate the enzyme to higher degree, or by the new enzyme biosynthesis. POMERANZ and SCHELLENBERGER (1961) investigated the release of fatty acids in germinated corn seeds by using Acrilidin orange indicator, they found that FFAs appeared in other new areas on the profile of seed indicating the transferance of enzyme activity during germination. On the other hand, DRAPRON and co-workers (1969) found that lipase activity decreased at the first steps of wheat seed germination, but the next steps showed higher increase in lipase activity. The authors explained this manner by the biosynthesis of new lipase at that period. MADYAROV and co-workers (1975) succeeded to extract and characterize several types of lipase during the germination of cotton seeds.

2.3. Fatty acid composition during germination

Table 2 shows that the changes took place on FA composition of germinated seeds. The percentage of palmitic, stearic and oleic acid in extracted oil samples decreased to some extent indicating that lipase of safflower seeds is specific to long chain saturated FAs since 35% of stearic acid, 14% of palmitic and 3.61% of oleic acid were lost during the process of germination.

The change in the concentration of linoleic acid was unremarkable, this depicts that lipase of this product is not specific to the higher degree of un-

Table 2

Changes in fatty acid composition of germinated safflower seed oil by GLC

Fatty acid	Changes of fatty acid composition (%) during germination periods (h)							Variation after 60 h (%)
	0	15	30	45	60	$\bar{x}$	$\pm s$	
C_{14}	0.15	0.14	0.14	0.13	0.13	0.14	0.01	—13
C_{16}	8.15	7.68	7.21	7.10	7.00	7.43	0.45	—14
C_{18}	3.10	2.17	2.15	2.10	2.00	2.30	0.59	—35
$C_{18:1}$	12.40	12.33	12.2	12.11	12.01	12.21	0.2	—3.6
$C_{18:2}$	76.20	77.68	78.3	78.46	78.86	77.90	0.93	+2.7

Fatty acid (%) is the w (%) of each fatty acid in 0.05 g of esterified safflower oil. For condition see the text.

$\bar{x}$: mean values; $\pm s$: standard deviation

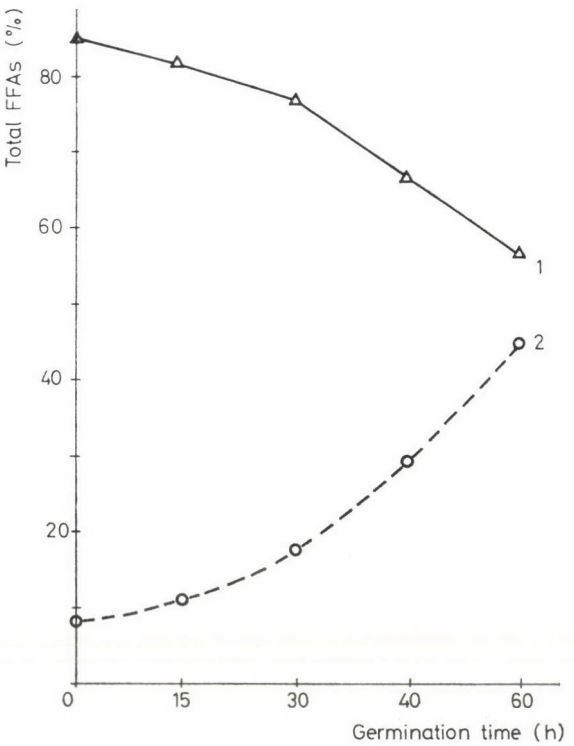

Fig. 5. Effect of germination on total FFAs content of safflower seeds. FFA (%) is the w % of total FFAs in 0.05 g of separated FFA fraction. 1: unsaturated FFAs; 2: saturated FFAs

saturation. VYAS and co-workers (1969) studied the changes taking place in peanut oil during germination and found that the enzyme preferred saturated fatty acid esters more than unsaturated ones.

2.4. Free fatty acid composition

GLC analysis of silicic acid chromatography separated FAs, indicated that remarkable changes have taken place in the composition of these acids in the germinated seeds. These results agree with those of FA composition analysis. The results shown in Fig. 5 and Fig. 6 clearly reflect the specificity of safflower lipase. Total saturated FAs increased typically with the increase in lipase activity which is shown in Fig. 4, while unsaturated FAs decreased gradually up to 30 h then a great decrease took place indicating that biological oxidation processes are being activated at this stage of germination. Because of being the main saturated fatty acid, the curve of the changes in palmitic acid is similar to the curve of the changes in total saturated FAs. Stearic acid increased from undetectable amount to 4.5%, this reveals the preference of

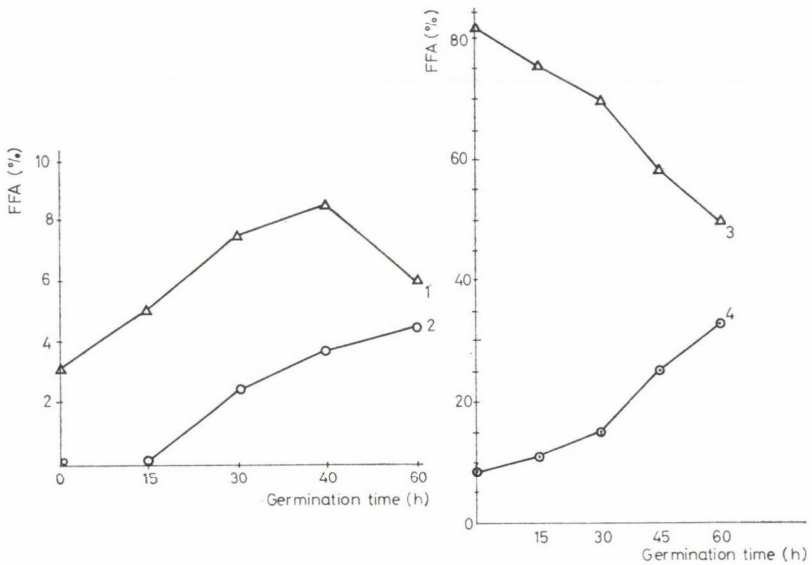

Fig. 6. Effect of germination on FFAs composition of safflower seeds. FFA (%) is the w % of total FFAs in 0.05 g of separated FFA fraction. 1: oleic acid; 2: stearic acid; 3: linoleic acid; 4: palmitic acid

safflower lipase towards the esters of this acid. Oleic acid increased up to 45 h of germination, then decreased, this may be due to the biological oxidation and decomposition occurring during this stage of germination. Linoleic acid oxidation and cleavage activity are well known in plant seeds, lipoxygenase and hydroperoxide isomerase and lipase have been well studied and characterized so the decrease in linoleic acid occurred during germination of safflower seeds may be due to the activity of these enzymes. NECHAEV (1972) investigated the changes in FA composition of oat seeds during germination, he found that saturated FAs increased remarkably, while linoleic acid concentration decreased because of the activity of lipoxygenase.

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DETECTION AND IDENTIFICATION OF *STAPHYLOCOCCUS AUREUS* IN FOOD BY SPECIFIC INHIBITION OF HEAT-STABLE DEOXYRIBONUCLEASE (TDNase) ACTIVITY

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A new technique is described for the detection and identification of *Staphylococcus aureus (S. aureus)* in food. The method is simple, rapid and specific. For the detection and identification of *S. aureus*, cultivation in proteose peptone – yeast extract (PPYE) and proteose peptone – yeast extract – 5% NaCl (PPYE NaCl) broths and TB-DNA-SS agar, containing specific anti-DNase staphylococcal immune serum and TB-DNA-NS agar, containing normal rabbit serum were used. The procedure allows the identification of thermo-stable deoxyribonuclease (TDNase) activity other than that of *S. aureus* origin. With the use of the method, depending on the degree of *S. aureus* contamination, the presence of *S. aureus* in food can be determined in 5–24 hours and from the time needed for the detection of TDNase, the initial quantity of *S. aureus* in food can be estimated.

Keywords: *Staphylococcus aureus*, deoxyribonuclease activity, inhibition of *Staphylococcus aureus*

Several procedures have been developed in the practice of food microbiology for the detection of *S. aureus* (BAIRD-PARKER, 1969; THATCHER & CLARK, 1968; MINOR & MARTH, 1971; ICMSF, 1974; NISKANEN, 1977; BOUWER-HERTZBERGER et al., 1982; ORMAY, 1984). The detection of *S. aureus* in foodstuffs with traditional methods requires 1–2 days and together with the identification of the strains, 2–4 days. Thus, efforts to shorten this time so as to give opinions about possible staphylococcal infection of foods, are desirable.

According to literature data, 99% of the *S. aureus* strains and 2% of other staphylococci produce TDNase (LACHICA et al., 1969; LACHICA et al., 1971; BARRY et al., 1973). Besides the staphylococci, certain bacteria belonging to the genera Streptococcus or Bacillus produce TDNase (TATINI et al., 1976; PARK et al., 1980; BECKER et al., 1984). It has been demonstrated that immune serum against *S. aureus* DNase can inhibit only the latter's activity and does not react with TDNase produced by other bacteria (BECKER et al., 1984; SCHARMANN & BLOBEL, 1968; LACHICA et al., 1979).

The purpose of the present investigation was to elaborate, based on these factors, a procedure for the detection of *S. aureus*, simpler and faster than the known methods and, at the same time, highly reliable.

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1. Materials and methods

1.1. Strains

A strain of *S. aureus*, isolated from a specimen of brawn, involved in an outbreak of food intoxication, was used in this investigation. The strain produced type A enterotoxin. *S. aureus* strain Wood 46 (OKI 10 004) was applied for the production of TDNase.

1.2. Food samples

A variety of commercially available foodstuffs was used. They showed different initial bacterial colony counts: UHT milk (< 10 cm^{-3}), pasteurised milk (10^2 cm^{-3}), cumin cheese (2×10^3 g^{-1}), brawn I. (8×10^3 g^{-1}), brawn II. (4×10^5 g^{-1}), brawn III. (6×10^5 g^{-1}) and vanilla ice cream (1.2×10^6 g^{-1}).

1.3. Media

— 3% proteose peptone (Difco) + 0.5% yeast extract (Difco), pH = 7.2 (PPYE)

— 3% proteose peptone (Difco) + 0.5% yeast extract (Difco) + 5% NaCl analytical grade (Finomvegyszer Szövetkezet, Budapest), pH = 7.2 (PPYE NaCl)

1.4. Culture methods

Foods were inoculated in a range of 10^2–10^7 cfu g^{-1} with *S. aureus*. From every food sample 1 g was added to 100 cm^3 Erlenmeyer flask containing 9 cm^3 of PPYE or PPYE NaCl media, in 3 replicates. The flasks were placed on an incubator shaker (about 100 r.p.m.) (Model G-24, NBS) and incubated at 37 °C. After 3, 5, 7 and 24 h incubations 0.5 cm^3 from the culture was heat-treated in a 100 °C water-bath for 15 min.

1.5. Production of specific anti-deoxyribonuclease staphylococcal immune serum (anti-DNase)

S. aureus strain Wood 46 was grown in PPYE broth at 37 °C for 24 h in an incubator shaker. After incubation, cells were harvested by centrifugation at 10 000 r.p.m., for 30 min, at 4 °C. DNase was produced from the supernatant according to FUCHS and co-workers (1967), except for using Amberlite CG-50 (Serva) cation exchanger resin instead of the initially applied phosphoryl-cellulose. About 2 kg body weight white rabbits were immunized with the DNase. For immunization, 100 μg DNase was dissolved in 0.5 cm^3 0.85% NaCl and mixed with complete Freund adjuvant (Difco). The suspension was injected s.c. in rabbits. After a week 200 μg DNase was injected, subsequently, at weekly intervals, the following quantities of DNase were administered: 400, 800, 1600, 3200 μg. A week after the last injection rabbits were bled to death. The produced immune serum was divided into 1 cm^3 portions and stored at −20 °C until use.

1.6. Detection of TDNase

For the detection of TDNase Toluidine Blue-DNA agar (TB-DNA) (LACHICA et al., 1971) was used.

The following procedure was elaborated for the detection of TDNase activity and for the identification of *S. aureus*. Two portions of 10 cm^3 TB-DNA agar were melted in test tubes and tempered to 50–53 °C. 0.1 cm^3 specific anti-DNase immune serum was added to one of the tubes (TB-DNA-SS agar), and 0.1 cm^3 normal rabbit serum to the other tube (TB-DNA-NS agar). After thorough mixing, the mixtures were poured into Petri dishes. After the agar had solidified, wells of 3 mm diameter were punched in it. From the heat-treated cultures a 10 μl quantity was applied to the wells of both TB-DNA-NS and TB-DNA-SS agar plates. These were incubated at 37 °C and after 1, 2, 3 and 4 h the diameters of the TDNase activities represented by pink halos around the wells were determined by the use of a slide-gauge to ± 0.1 mm accuracy. The result was considered positive when three repetitions of each sample gave the same results. On a TB-DNA-NS and/or on a TB-DNA-SS agar plate, the parallel examination of 31 samples was possible.

2. Results

The results of examinations of pasteurised milk, UHT milk, cumin cheese and brawn I. samples (total colony count 10^2–10^3 g^{-1}) are shown together in Table 1. No effect of these substrates on detection times was observed, so that the results could be pooled.

These data demonstrate that when 10^2 cm^{-3} *S. aureus* was inoculated with food into PPYE and PPYE NaCl media, after 24 h of incubation in broth and after 1 h incubation in TB-DNA agar, the presence of TDNase could be detected. With a 10^3 cm^{-3} or 10^4 cm^{-3} *S. aureus* colony count, TDNase could be detected after 7 h incubation in broth and 2 h and 1 h incubation, respectively in TB-DNA agar. The time required for detection of TDNase decreased further when the *S. aureus* colony count was 10^5–10^6 cm^{-3}, respectively. In this case, even 5 h incubation in broth and 2 h and 1 h incubation, respectively, in TB-DNA agar proved to be sufficient for the detection of TDNase. Finally, with 10^7 cm^{-3} colony count, we obtained positive results in 5 h (3 h incubation in broth and 2 h incubation in TB-DNA agar).

In addition, samples of vanilla ice cream, brawn II and III were examined (total colony count 10^5–10^6 g^{-1}). Results are summarized in Table 2, once again these substrates did not affect detection times in different ways.

The data show that when 10^2–10^3 cm^{-3} *S. aureus* was inoculated with food into the media, after 24 h incubation, only samples from the PPYE NaCl cultures showed TDNase activity. We suppose that the competitive flora considerably inhibited the proliferation of *S. aureus* in the PPYE medium whilst

Table 1

Detection of TDNase activity of S. aureus *in pasteurized milk, UHT milk, cumin cheese and brawn I*

Colony count of *S. aureus* of food origin in the medium	Medium	Incubation time (h) of liquid medium															
		3				5				7				24			
		Incubation time (h) of TB–DNA plate				Incubation time (h) of TB–DNA plate				Incubation time (h) of TB–DNA plate				Incubation time (h) of TB–DNA plate			
		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
10^2 g^{-1}	PPYE	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+
	PPYENaCl	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+
10^3 g^{-1}	PPYE	−	−	−	−	−	−	−	−	±	+	+	+	+	+	+	+
	PPYENaCl	−	−	−	−	−	−	−	−	±	±	+	+	+	+	+	+
10^4 g^{-1}	PPYE	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
	PPYENaCl	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
10^5 g^{-1}	PPYE	−	−	−	−	±	+	+	+	+	+	+	+	+	+	+	+
	PPYENaCl	−	−	−	−	±	±	+	+	+	+	+	+	+	+	+	+
10^6 g^{-1}	PPYE	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
	PPYENaCl	−	−	−	−	±	+	+	+	+	+	+	+	+	+	+	+
10^7 g^{-1}	PPYE	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	PPYENaCl	∓	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2

Detection of TDNase activity of S. aureus *in vanilla ice cream, brawn II and III*

Colony count of *S. aureus* of food origin in the medium	Medium	Incubation time (h) of liquid medium															
		3				5				7				24			
		Incubation time (h) of TB–DNA plate				Incubation time (h) of TB–DNA plate				Incubation time (h) of TB–DNA plate				Incubation time (h) of TB–DNA plate			
		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
10^2 g^{-1}	PPYE	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	PPYENaCl	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+
10^3 g^{-1}	PPYE	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	PPYENaCl	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+
10^4 g^{-1}	PPYE	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+
	PPYENaCl	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
10^5 g^{-1}	PPYE	−	−	−	−	−	−	−	−	±	+	+	+	+	+	+	+
	PPYENaCl	−	−	−	−	−	−	−	−	±	±	+	+	+	+	+	+
10^6 g^{-1}	PPYE	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
	PPYENaCl	−	−	−	−	±	+	+	+	+	+	+	+	+	+	+	+
10^7 g^{-1}	PPYE	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	PPYENaCl	±	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+

in PPYE NaCl medium *S. aureus* was able to proliferate and produce TDNase (GRAVES & FRAZIER, 1963; TROLLER & FRAZIER, 1963a, 1963b; PETERSON et al., 1964; IANDOLO et al., 1965). This was proved to be the case indeed by subcultures made from the incubated media. When the *S. aureus* colony count was 10^4 cm^{-3}, after 7 h incubation in broth and 2 h incubation in TB-DNA agar, the presence of TDNase could be detected using PPYE NaCl medium. After 24 h incubation, samples taken from PPYE and PPYE NaCl media were positive for TDNase. In the case of a colony count of 10^5 cm^{-3}, after 7 h incubation all samples were positive. With a colony count of 10^6 cm^{-3}, after 5 h incubation and with a count of 10^7 cm^{-3} after 3 h incubation in broth and 2 h incubation in TB-DNA agar, a pink halo indicating the presence of TDNase could be seen around the wells.

3. Discussion

According to our former investigations (MAJOR & EZEPCHUK, 1982) from among the liquid media tested, PPYE broth appeared to be the most convenient one for the detection of the activity TDNase produced by *S. aureus*. In the present study, however, examination of different foods showed that though in essence PPYE medium ensures favourable circumstances for the proliferation of *S. aureus* and for TDNase production, when the competitive flora of the food outnumbers *S. aureus* by some orders, it may happen that even after 24 h incubation, it is impossible to detect the presence of TDNase (Table 2). Therefore to suppress the proliferation of the competitive flora in foodstuffs to some extent, PPYE medium was made selective by the addition of 5% NaCl. As can be seen in Table 2, when using PPYE NaCl medium TDNase activity was detected when it could not be demonstrated in PPYE medium.

An additional advantage of PPYE NaCl is that 5% NaCl inhibits the activity of proteolytic enzymes which accumulate in the medium during cultivation and decrease the activity of TDNase enzyme (LACHICA et al., 1972). However, NaCl inhibits the growth of *S. aureus*, too – although to a considerably lesser degree. We observed e.g. that TDNase activity of the sample was lower when cultivated in PPYE NaCl medium than when cultivated in PPYE. Furthermore, it is known that the presence of salt in the medium exerts an inhibitory effect on the growth and proliferation of injured bacteria (FLOWERS & ORDAL, 1979; BUCKER et al., 1979). Therefore according to the above reported results and literature data, the parallel use of the two liquid media seems expedient for the detection of *S. aureus* from foods and also end results on TB-DNA-SS and TB-DNA-NS agar plates are to be compared. When a positive result is obtained in TB-DNA-NS agar (pink halo

around the sample containing well) whereas in TB-DNA-SS agar no TDNase activity can be observed, then the sample contains TDNase produced by *S. aureus*. But when the pink halos of the same diameter can be seen around the corresponding wells of both agar plates, then the heat-treated sample does not contain *S. aureus* TDNase. This phenomenon can be explained by the

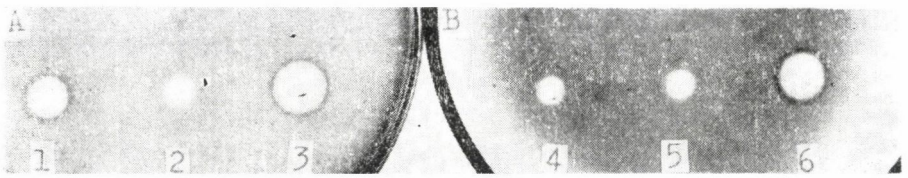

Fig. 1. Application of TB-DNA-NS (A) and TB-DNA-SS (B) agar plates, respectively, for the detection of TDNase activity. 1 and 4: TDNase activity of *S. aureus*, 2 and 5: negative control (PPYE medium), 3 and 6: TDNase enzyme activity other than of *S. aureus* origin

fact that the specific anti-DNase immune serum in TB-DNA-SS agar inhibits the activity of *S. aureus* TDNase and does not affect the activity of TDNase other than that of *S. aureus* origin (Fig. 1). Finally, it may be supposed when beside of *S. aureus* TDNase, TDNase of other than that of *S. aureus* origin is present in the culture, then results will be positive on both agar plates but the activity dependent halo will be greater in TB-DNA-NS agar then in TB-DNA-SS agar. The cause of this is that in TB-DNA-NS agar the activities of both strains are accumulated whilst in TB-DNA-SS agar only that of other organisms than *S. aureus* can be seen. Applying this procedure the presence of *S. aureus* can be more rapidly detected in foods than with traditional methods. Especially in examinations aiming at the detection of *S. aureus* food intoxications can be obtained quick and reliable results. Namely, a 10^6–10^8 g^{-1} *S. aureus* colony count is needed for the accumulation of that quantity of enterotoxin which causes food intoxication. As the results show, in such cases 5 and/or 3 h incubation in broth and 1–2 h incubation in TB-DNA-SS and TB-DNA-NS media, respectively, suffice to obtain positive results. A further advantage of the method is that when using TB-DNA-SS and TB-DNA-NS agar plates, respectively, further identification procedures are not needed because the resulting TDNase activity or activity-inhibition shows clearly whether *S. aureus* is present in the food.

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DOES THE USE OF FERTILIZERS AND PESTICIDES IN PLANT PRODUCTION IMPAIR THE WHOLESOMENESS OF FOODSTUFFS OF ANIMAL ORIGIN?*

(A REVIEW)

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The chemical composition of plants and their fruit is genetically determined, but also influenced by climate, soil quality, cultivation methods, degree of ripeness and other factors. The use of fertilizers and pesticides can also affect certain plant constituents; e.g. nitrate levels in certain plants can be considerably elevated by excessive use of nitrogen fertilizers. In the past, application of persistent pesticides has led to undesirable residue levels in foods and feedstuffs. With currently allowed non-persistent pesticides this problem does not occur. Death-rates from cancer in general or from cancer of the stomach are not correlated with usage of nitrogen fertilizers. Life expectancy of newborns has doubled in the last 100 years. In recent years, life expectancy has continued to increase both for women and men. Speculations that intensive farm production has undesirable effects on public health are not based on fact.

Keywords: fertilizers, pesticides, foodstuffs of animal origin, wholesomeness of foodstuffs

People live longer nowadays yet they are more concerned about their health. Frequently the question arises whether the quality of meat, milk and eggs might be adversely affected by currently practised intensive farm production. One aspect of this question will be dealt with here: possible effects of the use of mineral fertilizers and pesticides on the health quality of foodstuffs of animal origin. The majority of my remarks are related to the situation in the Federal Republic of Germany; however, with certain reservations, these remarks also apply to all other countries with intensive farm production.

1. Use of fertilizers

Figure 1 illustrates that from 1879 to 1979 the nutrient input per hectar of cultivated area has increased by more than ten times. This increase is not only due to the use of commercial fertilizers, but also to the increase in animal population which made a larger quantity of farm manure available. The term

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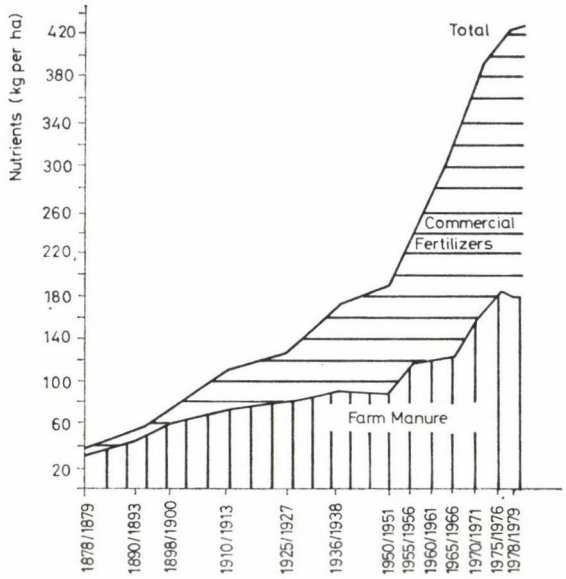

Fig. 1. Consumption of commercial fertilizers and farm manure in the Federal Republic of Germany and the German Reich, respectively. Supply of nutrients (sum of N, P_2O_5 and K_2O) per hectar of cultivated area (KUNTZE & VOSS, 1980)

farm manure signifies animal excrements and residues of feeds and litter, including liquid and semi-liquid manure, but not including substances of purely vegetable origin like straw, green manuring and compost produced without animal excrements.

In view of this enormous increase (which apparently has not continued after 1981 – see Fig. 2 on commercial nitrogen fertilizers) one may well wonder what this will lead to. One very positive result is the increase in crops. In the middle ages 1 kg of grain seeds yielded 3 to 4 kg of grain. Nowadays, the yield on average soil is 40 kg, on good soil 70 kg and more. But does the enormous increase in nutrient input not change plant composition and hence plant quality? On the one hand, the chemical composition of plants and their fruit is genetically determined; on the other hand it is influenced by the degree of ripeness, the climate, soil quality, cultivation technique (plant spacing, irrigation, plant protection) and fertilizing. Many people have wrong ideas about the nature and the extent of the effect of using fertilizers. Already in 1935, JACOB wrote: "Urban consumer circles often voice the fear that, although yields can be increased by the use of commercial fertilizers, both quality and wholesomeness of the final products suffer. Nobody was ever able to prove this, but it is well known that, in the matter of health, people will believe almost anything, especially when such arguments are supported by the slogan 'back to nature'." Research carried out since that time showed that many

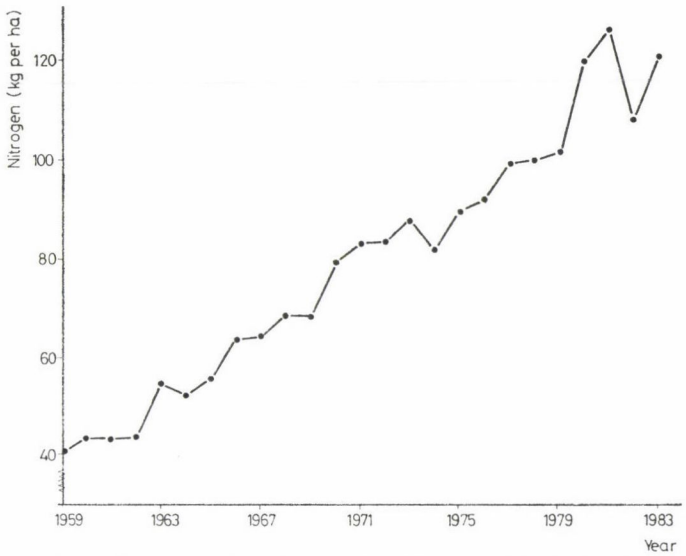

Fig. 2. Consumption of commercial nitrogen fertilizers in the Federal Republic of Germany, per hectar of cultivated area (BUNDESMINISTER FÜR ERNÄHRUNG, LANDWIRTSCHAFT UND FORSTEN, 1984)

plant constituents are relatively little affected by fertilizing. This is true e.g. for many vitamins. Carotene, which, in some plants, can be considerably enriched by fertilizing with nitrogen and potassium, is an exception. Table 1 illustrates that optimal use of P and N increased the carotene content of perennial ryegrass by the factor 5.

Of course the cultivation of soil which is poor in minerals can result in crops with low mineral contents, and fertilizing can increase these contents. Such a result is shown in Table 2: the use of farm manure increased the potassium content, the use of NPK-fertilizers (nitrogen, phosphate, potassium) improved the potassium and phosphate content of potatoes. The use of NPK plus farm manure resulted in a slight additional increase of the potassium content and brought about no further increase in the phosphate content.

Table 1

Influence of various degrees of fertilizing with nitrogen and potassium on the carotene content of perennial ryegrass
(SCHARRER & BÜRKE, 1953)
(mg carotene per 10 g dry matter)

Manuring	P_0	P_1	P_2	P_3
N_1	1.53	2.03	2.33	2.09
N_2	2.15	4.16	5.21	5.06
N_3	2.14	6.87	7.54	7.83

Table 2

Effect of manuring on the composition of potatoes
(NEHRING, 1965)

Manuring	Yields (dz per ha)[a]	N (%)	K_2O (%)	P_2O_5 (%)	Starch (%)
		1958 (wet year)			
Not manured	62	1.48	1.61	0.31	18.7
NPK	354	1.14	2.26	0.58	17.7
Farm manure (300 dz per ha)	202	1.28	2.17	0.32	19.5
NPK + farm manure	389	1.45	2.47	0.52	17.8
		1959 (dry year)			
Not manured	73	1.67	2.00	0.39	18.7
NPK	386	1.04	2.37	0.53	19.0
Farm manure (300 dz per ha)	329	1.44	2.48	0.40	18.9
NPK + farm manure	437	1.31	2.62	0.51	18.3

[a] 1 dz = 100 kg

Table 3

The use of different types of fertilizer has no effect on the starch and vitamin C content of potatoes
(KLETT, 1968)

	Vitamin C content of potatoes (mg per 100 g fresh matter)		
	1965	1966	1967
Growth in the shade			
use of bio-dynamic manure	20.1	22.2	17.3
use of mineral fertilizer	19.7	20.9	19.3
Growth in full daylight			
use of bio-dynamic manure	33.9	39.7	18.8
use of mineral fertilizer	38.2	34.7	18.9

	Starch content of potatoes (g per 100 g fresh matter)		
	1965	1966	1967
Growth in the shade			
use of bio-dynamic manure	19.3	10.1	11.8
use of mineral fertilizer	18.3	10.3	12.9
Growth in full daylight			
use of bio-dynamic manure	24.1	15.4	15.9
use of mineral fertilizer	22.5	16.0	14.3

The question whether there are differences in quality due to the use of organic or mineral fertilizers has, for decades, been the subject of animated discussions between the representatives of modern and "alternative" farming. From a scientific point of view these differences – if there are any – are small. This is illustrated in Table 3 by the vitamin C and starch content of potatoes cultivated either by using "bio-dynamic" compost or by using an equivalent quantity of mineral fertilizer. The plants stood either in full light or in the shade. Whereas light and climatic differences from one year to the next had a noticeable effect on the vitamin C and starch content, the use of one or the other type of fertilizer had no distinct influence.

The protein content of many plant products is of considerable importance. The breadmaking quality of wheat is largely dependent on a sufficiently high protein content. Barley, if used in beer production, should have a low protein content; this also applies to sugar beets in sugar production. In feedingstuffs, a high protein content is desirable; this not only refers to the quantity but also to the composition of proteins, i.e. their content in essential amino-acids. The effect of fertilizing on protein quantity and quality was examined with particular attention, and the results were rather contradictory. As is illustrated in Table 4, manuring in a wet year resulted in an increase, whereas in a dry year it led to a decrease in the crude protein content (% N) of summer barley. This makes it quite clear, in addition to what is illustrated in Tables 3 and 4, that the results of only one crop year are not conclusive. Such studies have to be continued over several years.

The choice of cultivar is another factor that may influence the results of such studies. As reported by EL-NEGOUMY and co-workers (1982), increasing levels of nitrogen fertilizer led to increased total protein in several barley cultivars, but not in the cultivar Hiproly. That crop composition can also be

Table 4

Influence of manuring on the composition of summer barley (NEHRING, 1965)

Manuring	Yields (dz per ha)	N (%)	K_2O (%)	P_2O_5 (%)
1958 (wet year)				
Not manured	15.2	1.66	0.80	1.08
NPK	25.6	1.74	0.75	1.00
Farm manure (300 dz per ha)	26.4	1.73	0.74	1.04
NPK + farm manure	32.0	2.07	0.87	1.11
1959 (dry year)				
Not manured	11.2	1.62	0.68	0.88
NPK	31.4	1.50	0.47	0.72
Farm manure (300 dz per ha)	32.2	1.49	0.60	0.85
NPK + farm manure	40.9	1.49	0.63	0.89

influenced by one-sided manuring, e.g. by using only nitrogen fertilizers, is shown by the following experiment: winter wheat was grown either without manuring or by using 160 g of urea per m². According to Table 5, flour made

Table 5

Influence of fertilizing with nitrogen on various properties of wheat flour
(TIMMS et al., 1981)

	Without fertilizing	With 160 g urea per m²
Sulphur (g per kg dry matter)	2.3	2.45
Nitrogen (g per kg dry matter)	17.4	21.2
Relation (S : N)	0.132	0.116
Baking volume (cm³)	174	191

from wheat which was cultivated with nitrogen fertilizer showed better baking quality (higher baking volume). The nitrogen (protein) content had increased more than the sulphur content as indicated by the lower S/N ratio. Amino acid analysis showed a distinctly lower level of the sulphur-containing amino acids: cystine and methionine (Table 6). In this case, the one-sided use of nitrogen fertilizer led to a sulphur deficiency in the wheat proteins. Other studies on the effect of nitrogen fertilization on wheat proteins have been reported by POMERANZ and co-workers (1976), MCGUIRE and co-workers (1979),

Table 6

Influence of fertilizing with nitrogen on the composition of the amino acids of the protein in wheat flour
(TIMMS et al., 1981)

	not fertilized	fertilized	not fertilized	fertilized
	μmol per g flour		mol per 1000 mol	
Aspartic acid	32.3	49.6	43.2	49.3
Threonine	23.5	28.5	31.4	28.3
Serine	48.5	62.8	64.8	62.4
Glutamic acid	231.1	315.4	309.0	313.5
Proline	117.3	150.3	156.8	149.4
Glycine	45.3	62.6	60.6	62.2
Alanine	32.6	47.1	43.6	46.8
Cystine	19.8	20.6	26.5	20.5
Valine	28.2	41.0	37.3	40.8
Methionine	11.3	10.7	15.1	10.6
Isoleucine	21.4	29.0	28.6	28.8
Leucine	50.3	60.3	67.2	59.9
Tyrosine	18.8	25.4	25.1	25.2
Phenylalanine	22.8	35.0	30.5	34.8
Histidine	16.1	24.5	21.5	24.4
Lysine	13.3	15.8	17.8	15.7
Arginine	14.9	26.4	19.9	26.2

and DOEKES and WENNEKES (1982). As reported by VAUGHAN and co-workers (1980) protein contents of all varieties of rice which they studied increased with fertilizer level.

If different forms of manuring have such a noticeable influence on plant constituents like carotene and protein, which are nutritionally important, then the nutritional value of foodstuffs and feedingstuffs can certainly be influenced by manuring. The question we are particularly interested in relates to possible effects on consumer health. Can fertilizing, aimed at maximizing yields, lead to the formation of harmful substances? Is there any direct danger (by eating fertilized plant food) or indirect danger to our health (by eating food produced by animals which received feedingstuffs from intensive farm production)?

Many analytical tests carried out with plants fertilized in different ways give no indication of any formation of harmful substances. As regards oxalic acid in spinach and solanine in potatoes some studies have been carried out in our laboratories (DIEHL & WEDLER, 1978). In nitrate-storing plants, like spinach, red beet, lettuce, endive, radishes, red radishes, etc., the nitrate content can be considerably increased by N-manuring. Nitrate as such is harmless, but in the human organism it is partly changed into nitrite by bacterial action. Babies under 3 months react very sensitively to nitrite (methaemoglobinaemia, cyanosis). In vitro experiments have shown that nitrite and amines can react to nitrosamines, which in animal tests were proven to be carcinogenic. To what extent nitrosamines can be formed in the human body, and whether endogenous formation of nitrosamines can increase the risk of cancer for human beings has not been established. However, as a precaution, some scientists and organizations recommend the avoidance of a high intake of nitrate. This also means that intensive use of nitrogen fertilizers should be avoided with the above mentioned nitrate-storing plants. Some governments, e.g. Switzerland, have recently set tolerance levels for nitrates in vegetables. In examining the wholesomeness of feedingstuffs, we must also remember that nitrate – or rather the nitrite formed from nitrate by bacterial action – can be toxic to animals (WRIGHT & DAVISON, 1964). However, a high nitrate content in feedingstuffs does not lead to a significantly higher nitrate content in meat, milk and eggs. The average daily intake of nitrate (without drinking water) per head in the Federal Republic of Germany is about 75 mg. About three fourths of this stem from vegetables. Foodstuffs of animal origin contribute very little. Cow's milk for example contains on average less than 0.4 mg of nitrate per dm^3 (DFG, 1983).

Not nearly all constituents of feedingstuffs and foodstuffs have been determined by chemical analysis. It is therefore of interest to know whether animal feeding tests give any indication of the presence of harmful substances stemming from fertilized feedingstuffs. Already 50 years ago SCHEUNERT and

co-workers (1934) carried out extensive feeding trials with rats; no differences were found between feeds produced with mineral fertilizers and those produced with organic manure. In contrast, HAHN and co-workers (1971) described feeding trials carried out with rabbits, which seemed to indicate fertility defects following the intake of feeds produced with mineral fertilizers. The authors assumed that "increasingly intensive farm production leads to the occurrence or the activation of organic plant constituents hitherto unknown, which negatively affect sexual functions". However, the experimental method used by these authors leaves room for doubts concerning the results and their interpretation (DIEHL & WEDLER, 1978). Since then, feeding tests carried out at the University of Giessen with rabbits (MEINECKE, 1982) and at our own research institute with mice and rats (NEUDECKER & WEDLER, 1983; NEUDECKER et al., 1982) showed no such results following the intake of plant feeds which had been grown with mineral fertilizer.

From the available literature we must conclude there are no indications that fertilizing, even when aimed at maximal yields, produces feedingstuffs, the use of which would negatively affect the wholesomeness for human beings of food of animal origin. There are other valid reasons why fertilizing should not be exclusively oriented towards maximizing yields, e.g. in order to avoid an increase in the nitrate content of ground water.

On the other hand, it can be seen that fertilizing which is scientifically sound and adapted to the needs of the plants will produce healthy feedingstuffs, which more than ever enable farmers to produce healthy animals. I should like to give two examples. In the old days, cereals grown on moor and moor sand were often affected by the "moor disease". In May and June, the edges and the tips of the leaves dried out. In serious cases, no ears were formed. The cause was a copper deficiency in these soils. Today, this problem can be avoided by using fertilizers which contain copper. This is also good for the animals. Feed grains grown on soils which are low in copper show a low copper content. If such feeds are being used over a long period of time, the animals can develop copper deficiency, the symptoms of which are licking disease, loss of weight and anaemia. Since fertilizers containing copper were used in these regions, this animal disease has disappeared. The second example refers to another deficiency disease, which until the end of the 50s had disastrous consequences for cattle rearing in certain areas of the Black Forest and in other regions, the soils of which lie on granite. The animals also showed licking disease, anaemia, loss of weight and loss of vitality. It was hardly possible to bring up young animals in these regions. Research work carried out at the Agricultural Research Institute Augustenberg (Karlsruhe, FRG) showed that a lack of cobalt in these soils resulted in a cobalt deficiency in feed plants and thus in a cobalt deficiency in animals. The problem was solved by using fertilizers with added cobalt.

2. Use of pesticides

Public discussions about the advantages and disadvantages of the use of pesticides mostly refer to insecticides, although in European countries these are used in much smaller quantities than fungicides or herbicides – see Fig. 3. However, the conditions in tropical regions are different. In India, for example, 74% of all plant protection products used in 1980 were insecticides, 18% were fungicides and only 4% were herbicides (MUKERJEE, 1982). The reason for the public interest in insecticides is the occurrence of undesirable ecological consequences of their use. They can destroy not only harmful but also useful insects. Insecticides used during the first decades after the Second World War, mostly belonging to the group of chlorinated hydrocarbons, showed great persistence in the environment. The best known representative of this group is DDT, which has many excellent qualities. It is not very toxic for humans and domestic animals and, owing to its persistence, a sole utilization per season was usually sufficient. By using this substance, malaria was eradicated in large areas (in Europe e.g. in Sardinia and South Italy) and the lives of millions of people were saved in countries which before were plagued by sleeping-sickness, spotted fever, yellow fever, typhoid fever and other epidemic

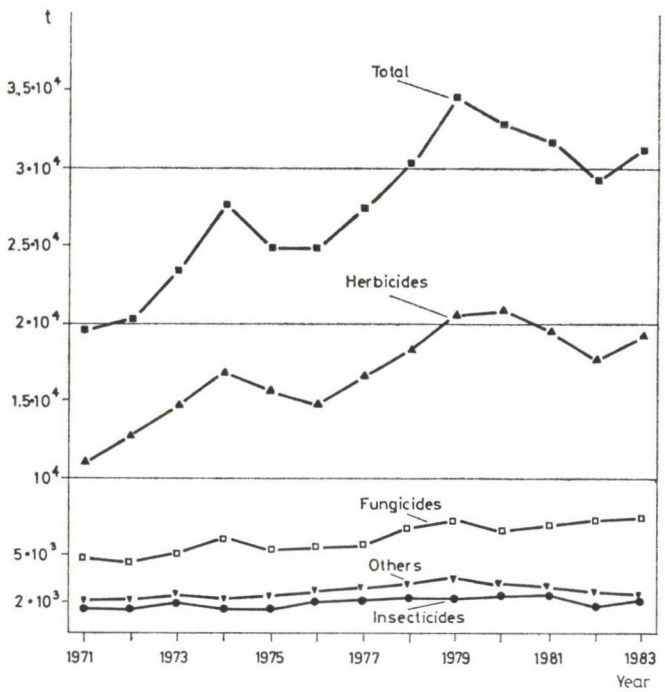

Fig. 3. Annual sales of pesticides (t per year) in the Federal Republic of Germany (BUNDESMINISTER FÜR ERNÄHRUNG, LANDWIRTSCHAFT UND FORSTEN, 1984)

diseases. Unfortunately, the persistence of DDT proved to be a boomerang. It spread in the environment, was found even in plancton in the oceans, in fish and birds. Increasing levels of DDT were also detected in human tissues and in mother's milk. Although there were no indications of injuries to health of human beings due to DDT intake via food – and until today there have been no such indications – these findings were alarming. In 1972 production and use of DDT were banned in the Federal Republic of Germany, and in the following years the other persistent chlorinated hydrocarbons were gradually

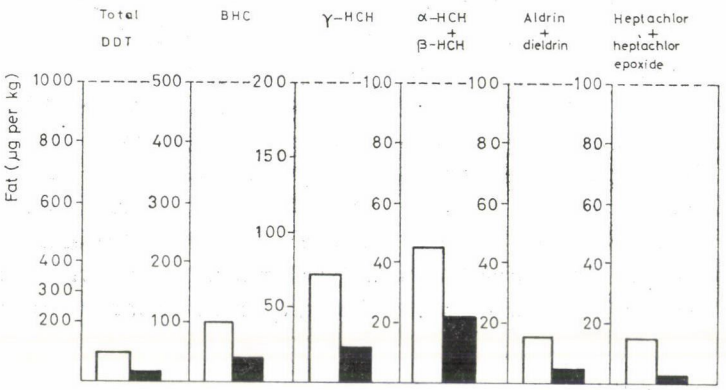

Fig. 4. Decrease of residues of chlorinated hydrocarbon pesticides in cow's milk 1974–1981 (DFG, 1983). □: 1974; ■: 1981; - - - -: permitted maximum level

withdrawn from the market. These bans resulted in a distinct decrease in the levels of residues in feedingstuffs and foodstuffs. This is illustrated in Fig. 4 with results of the analysis of milk. The graph also shows that the average levels of residues are far below the authorized maximum level. Individual samples may show considerably higher concentrations and may occasionally exceed the tolerance level. In recent years, this has happened rarely, and only in the case of α- and β-HCH.

The non-persistent insecticides, herbicides, fungicides, etc. which are authorized today, are of even less importance in foodstuffs of animal origin than in those of plant origin. These substances are largely decomposed in the interval between their application and the time of harvest. The low residual levels which get into the animal body via feedingstuffs are catabolized there or are excreted with the fecal matter or in the urine. In foodstuffs of animal origin, residues of such products can usually not be detected.

The reason why residues of chlorinated hydrocarbons can still be found in meat, milk and eggs, despite the ban of these products, is the persistence of these compounds, but also imports of feedingstuffs from countries which still allow the use of these pesticides. Various indicators show a link between the

Table 7

Residues in cow's milk and comparison of the daily intake with ADI levels, average 1977–1980
(DGE, 1984)

Substance	ADI level (μg per 60 kg body weight)	Concentration measured (mg per kg fat)	Intake with 1 dm³ milk (μg)	Intake (% of ADI)
HCB	36	0.05	1.7	5
α-HCH	300	0.04	1.4	0.5
β-HCH	60	0.02	0.7	1
Lindane	600	0.03	1.0	0.2
Heptachlorepoxide	30	0.01	0.3	1
Dieldrin	6	0.01	0.3	5
Total DDT	300	0.03	1.0	0.3

use of imported feedingstuffs and the residual levels in foodstuffs of animal origin. When unusually high levels of α- and β-HCH levels in milk were detected in recent years, this occurred mostly in Northern Germany, where more imported feeds are being used than in Southern Germany.

What might be the consequences of such residues in foods on our health? In order to answer this question, a comparison between the quantities taken in via food and the ADI levels (acceptable daily intake) set up by FAO/WHO is useful. The ADI level of a substance is the quantity which can be consumed daily and over a whole lifetime without having any negative effects on health. Table 7 illustrates that the consumption of 1 dm³ of milk by an adult with a

Table 8

Levels of chlorinated hydrocarbons in mother's milk and comparison of the intake of an infant during the first 4 weeks of life with the ADI level
(DFG, 1984)

Substance	ADI level (μg per kg of body weight)	Median values of concentration observed in mother's milk (mg per kg fat)	Concentration not causing intake to exceed ADI[a] (mg per kg fat)	Actual intake exceeds ADI by a factor of
HCB	0.6	1.17	0.12	10
α-HCH	5	0.012	1.03	—
β-HCH	1	0.25	0.21	1.2
Lindane	10	0.045	2.07	—
Heptachlorepoxide	0.5	0.025	0.10	—
Dieldrin	0.1	0.032	0.02	1.6
Total DDT	5	1.68	1.03	1.6

[a] Calculated under the following assumptions: average body weight 3.7 kg; average daily intake of mother's milk during the first 4 weeks of life 532 cm³ with 18 g of milk fat

body weight of 60 kg during the years 1977 to 1980 resulted in an intake of chlorinated hydrocarbons amounting to 5% or less of the ADI levels. Even when the residues in all other foods of the total diet are considered, ADI levels are not nearly reached. Due to the continued decrease in residue levels the situation is even more favourable today. Evaluations in other countries have led to the same conclusion. As one example, TURNBULL's (1984) report from the United Kingdom might be mentioned.

The situation is less satisfactory with breast-fed infants – on the one hand because the level of residues in mother's milk is higher than in cow's milk, on the other hand because the food intake related to body weight is much higher with infants than with adults. As indicated in Table 8, ADI levels are considerably exceeded in the case of HCB, and slightly exceeded in the case of β-HCH, dieldrin and total DDT. For several reasons this should not lead to the recommendation to mothers not to breast feed their babies: firstly, the analytical results on which Table 8 is based were obtained several years ago. The continued decline of chlorinated hydrocarbon levels in the environment and in mother's milk has improved the situation since then. Secondly, even some 10 years ago, when pesticide residue levels in mother's milk had reached maximum levels, no indications of ill effects were noted in breast-fed babies. Thirdly, ADI levels are calculated for life-time exposure, while mother's milk is consumed for only a few weeks or months. Nevertheless, the presence of such residues in mother's milk must be considered undesirable. Every possibility of further reducing the level of pesticide residues should be exploited. The animal feed industry can make an important contribution to this by not only respecting the maximum levels laid down in feed legislation but by using raw materials with the lowest possible level of residues.

Can the use of pesticides have an influence on the composition of plants? Such effects have indeed been noticed. The protein content of many plants can be increased by using the herbicide dichlorophenoxyacetic acid (2,4-D), as was found by ERICKSON and co-workers in 1948 with wheat and by PAYNE and co-workers in 1953 with potatoes. The carotene content of carrots can be increased by treating the soil with 1,3-dichloropropene (Telone), a fumigant for the control of nematodes (WU et al., 1970), and by using the herbicides linuron or chlorpropham (SWEENEY & MARSH, 1971).

According to WORTHINGTON and SMITH (1973), spraying groundnut plants with fungicides like Benlate (Benomyl) changes the composition of the fatty acids of the lipids of groundnuts. The content of stearic acid decreases, the content of linoleic acid increases. Soil fumigation with Nemagon increases the carotene and sugar content of maize (SALUNKHE et al., 1971).

Space does not permit discussion of many other studies carried out in this area. On the whole, the changes which were noted are small. If anything, they tend to be favourable (higher content of valuable nutritive substances),

rather than harmful. This research has not given rise to concern about wholesomeness.

Before closing the chapter on pesticides, I should like to point out that the non-persistent products which are currently used often present higher acute toxicity for human beings than the persistent products used formerly, such as DDT. Carelessness in the use of such products can lead to poisoning, even death, of persons handling such pesticides.

3. Health statistics

Critics of modern farming often maintain that products from intensive farm production are unhealthy. Are there any health statistics which illustrate that this is the case? Is there any truth in the assertion that intensive use of fertilizers increases the incidence of cancer of the stomach or – as some say – of cancer in general? The incidence of cancer of the stomach has greatly declined during the last decades in all European countries, while fertilizing has increased in intensity. Therefore, it is very unlikely that there should be a causal link between cancer of the stomach and the use of fertilizers. The overall risk of cancer is smaller in the Netherlands, where the quantities of fertilizers used per hectar are highest, than in countries like Austria or Great Britain, where less fertilizer is used (Fig. 5). It is often said that nowadays more young people die of cancer. The figures in Table 9 show that this is not the case – at least not in the Federal Republic of Germany. On the contrary, death-rates from cancer have remained remarkably stable in the different age groups.

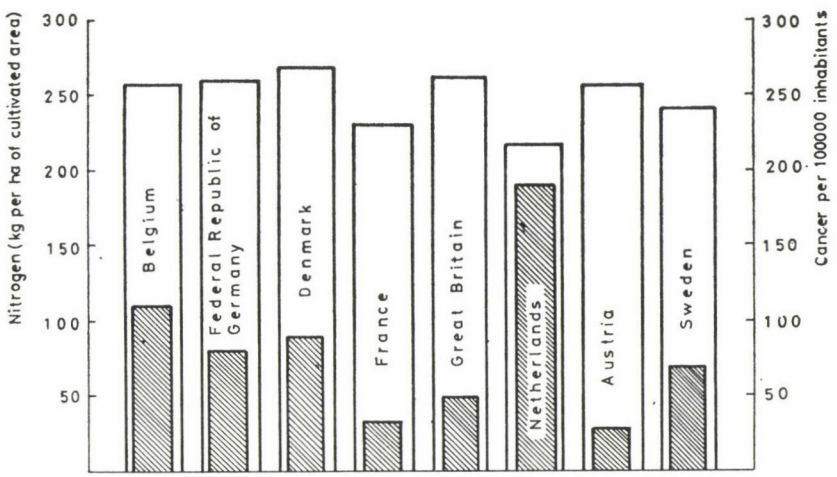

Fig. 5. Cancer death rate and use of commercial nitrogen fertilizers in European countries (KUNTZE & VOSS, 1980). ▨: Amount of nitrogen; □: number of deaths per 100 000 inhabitants

Table 9

Death rates from cancer in the Federal Republic of Germany. Mortality of various age groups
(BUNDESMINISTER FÜR JUGEND, FAMILIE UND GESUNDHEIT, 1983)

Year	Deaths from malignant tumors	
	out of 100 000 persons under 30 years of age	out of 100 000 persons age group 30–60 years
1950	6.8	129.5
1957	8.8	144.1
1962	8.8	143.5
1972	7.8	116.3
1977	6.9	117.3
1979	6.6	123.1
1981	6.1	122.2

More important than the incidence of various diseases or the causes of death is the overall mortality or the average life expectancy. Figure 6 contains data for Germany, which are similar in other European countries. According to this graph, the life expectancy of newborn babies has approximately doubled during the last 100 years. The life expectancy of thirty-year olds has increased

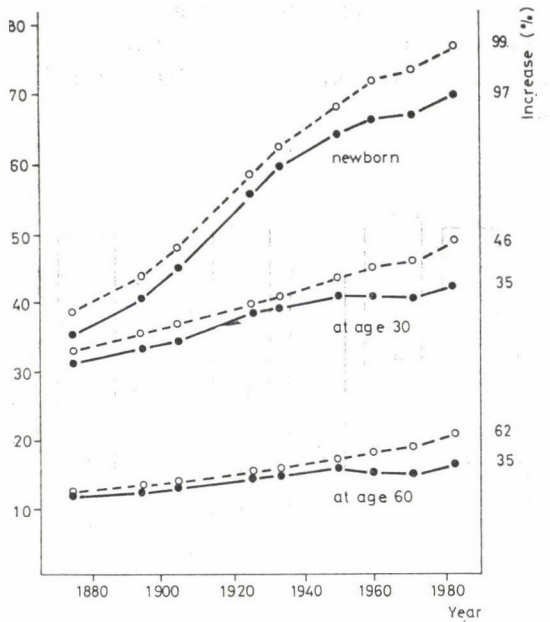

Fig. 6. Mean life expectancy of newborn infants and of 30 and 60-year-old men and women in the Federal Republic of Germany and the German Reich, respectively (Statistisches Bundesamt, 1984). ●——●: men; ○——○: women

by 34% (men) and 45% (women) compared to 100 years ago. The reason why the increase for men in the 1950s and 60s was not as continuous as with women is not known with any certainty even today. Some epidemiologists hold the view that excessive alcohol drinking and cigarette smoking, both more common with men, are responsible for this difference. At any rate, as men and women eat the same food, the quality of foodstuffs cannot be blamed for the lower life expectancy of males.

Neither these nor other statistical data, e.g. infant mortality or absence from work due to illness, indicate a deterioration of public health. Speculations that intensive farm production has undesirable effects on public health are not based on fact.

This does not imply that intensive farming may not have certain undesirable effects on the environment. Some problems caused, e.g. by monoculture or by over-fertilizing, are real enough. But the question posed at the beginning, does the use of fertilizers and pesticides in the production of plants impair the wholesomeness of food of animal origin, can clearly be answered in the negative.

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Acta Alimentaria, Vol. 15 (4), pp. 353—354 (1986)

BOOK REVIEWS

Food packaging and preservation. Theory and practice

M. MATHLOUTHI (Ed.)

Elsevier Applied Science Publishers, Ltd. London and New York, 1986, 402 pages

It is well-known that it is not enough to produce goods — one also needs to make sure that there is a market for them.To sell food product, their quality should be preserved as close as possible to that of the fresh or just-processed product and this can be achieved by controlling the packaging and preservation conditions.

In this book an attempt is made to gather the most recent information in the different fields related to food packaging and preservation by authors known for their expertise and their personal involvement in developing up-to-date research in their different fields.

It examines the problems posed by the stability and shelf-life of foodstuffs packaged in polymer materials and it is the first integrated presentation of the theoretical and practical aspects of food packaging and presentation.

The contributors of the book presented their papers at the First International Symposium on Food Packaging in France, held between May 20—23, 1985 at the University of Reims organized by ADRIAC (Champagne — Ardennes Area Food Industries Association), the University of Reims and Rhone-Poulenc Films. The Symposium consisted of six sessions and the book follows this system.

The first part covers the problems of mass transfer and permeability of polymer films regarding to the aspects of food packaging including the phenomena of migration. The topic of the second part is the consequences of water content changes on the microbiological stability of packaged food during storage. The third part gives a survey of water interactions and water activity during the preservation of packed foodstuffs with regard to their microbiological and organoleptic stability. The fourth part covers the problems of food stability including the shelf-life of packaged foodstuffs regarding to the water vapour and light permeability of packaging materials. The fifth part investigates the effects of surface structure and orientation of polymers used in food packaging to the quality changes of food. The main topic of the sixth part is the relationship between the packaging technologies and quality and quantity protection of packaged foodstuffs.

The book with 80 tables and 172 figures gives a good illustration of multidisciplinary packaging technique, therefore it is very useful for industrial specialists and for researchers.The First International Symposium organized by ADRIAC may be followed by others to continue the discussions between chemists, physicists, microbiologists, food technologists and packaging technologists.

I. VARSÁNYI

Food economy in Hungary

Ernő CSIZMADIA and Magda SZÉKELY

Akadémiai Kiadó, Budapest, 1986, 218 pages

The food economy is one of the most important sector in the Hungarian national economy. The object of the book is very actual both for the management and for the production.

Akadémiai Kiadó, Budapest
D. Reidel Dordecht

The authors give a good analysis of the development of the Hungarian food economy during the last decades.

The book consists of eight parts. Part one deals with the agrarian development in the first half of the 20th century, and gives a presentation of the rising standard of food production. This historical retrospection serves a correct evaluation of Hungarian agriculture's development of the past decades.

Part two deals with the development of agriculture and agrarian relations effected by changes in Hungarian society and economy. It gives a realistic picture of the role and significance of food economy and its interbranch relations.

Part three deals with the development and utilization of the forces of production. It gives a picture of the peculiar Hungarian landownership and leasehold relations, fixed and current asset management, the labour situation and the development of efficiency.

Part four presents abundant illustration of the interbranch structure in Hungarian food economy and the development of the main branches.

In part five enterprise structure is treated illustrating food processing plants, state farms, vertical manufacturing plants and agricultural cooperatives.

Part six introduces household and auxiliary farms and beside the dominant role of large-scale plants, the importance of small farms is discussed. The expected main trends of their development are outlined.

Part seven deals with joint ventures, cooperation and integration of enterprises. It treats enterprise partnerships established in the past decade, evaluating the main characteristics of their operation, and experience gained in the work of industry-like production systems and agrarian-industrial unions.

Part eight gives an overall picture of the economic management system of the food economy. It also deals with the Hungarian planning system, the sales and prices system, taxes and subsidies, credit policy, enterprise financing, profit regulation, wage system and wage policy.

Hungarian food economy is presented in a complex way, fitted into the entire system of the national economy. Surveying several decades it reveals and analyses the main trends of agrarian development, and outlines the perspectives of the eighties.

The book might be of interest not only for specialists but also for readers working in the field of agro-economic development.

I. VARSÁNYI

Advances in refrigeration and heat pump technology achieved by the application of micro-electronics and the control of systems by micro-electronic devices

International Institute of Refrigeration, Paris, 1984, 461 pages

The publication of 2 volumes and 461 pages contains the papers presented at the Conference of Commission B 2 of the International Institute of Refrigeration, held in Dresden (GDR), September 3—7, 1984. The conference treated a highly topical subject, the possibilities of applying micro-electronics in refrigeration and heat pump engineering and the trends of development. The conference was attended by more than 120 experts from 18 countries. The number of papers given in the plenary sessions and in five sections exceeded 50. The sections were as follows:

1. New developments, new solutions for components and installations created by the application of micro-electronics.
2. Comparison between conventionally automated components and installations with those controlled by micro-electronics.
3. Principles and methods for mathematically modelling the steady-state and dynamic behaviour of refrigeration components and installations.
4. Saving of energy by applying optimum conditions to steady-state and dynamic behaviour of refrigeration components and installations.
5. Data acquisition, data processing and the application of process computers to rationalize the work of man in refrigeration techniques and heat pump technology.

The above papers present useful informations even for experts interested in food refrigeration and the application and construction of refrigeration equipment.

GY. URBÁNYI

CARIES PREVENTION BY DOMESTIC SALT FLUORIDATION

By K. Tóth

In English. 249 pages, 15 figures. 14×21 cm.
Hardcover $18.00/DM 54,—/£14.50
ISBN 963 05 3476 2

In Hungary a series of clinical trials started in 1966 with the aim of testing the caries-preventive effect of fluoride added to domestic salt in different concentrations. Experiments were carried out paralell with three different salt-F concentrations and as a result caries fell by more than 50 per cent. Investigations confirmed furthermore that fluoridesupplemented salt has the same effect on deciduous teeth as fluoridated water.

In this book also the physiological aspects of salt fluoridation like daily optimal intake, tolerable and harmful amounts, drinking habits are dealt with. Finally the recent results reached in the field of caries prevention are assessed.

AKADÉMIAI KIADÓ
Publishing House of the Hungarian
Academy of Sciences
Budapest

RESEARCH ON DIETARY FIBRES

A Joint Study of Medicine, Nutrition and Industry

Edited by
Cs. Ruzsa, T. Jávor and Gy. Mózsik

Symposium on the Possibilities of the Chemical Determinations of the Dietary Fibres and their Nutritional and Dietetic Roles September 23–24, 1983, Pécs, Hungary

In English. 1986. XIV + 222 pages. 1 photos, 52 figures. 44 tables. 17×25 cm. Hardcover.
$24.00/DM58,—/£17.00
ISBN 963 05 4254 4

The basic changes in the living conditions of the Hungarian population during the past three decades resulted in an unfavourable tendency in nutrition. Researchers have detected that the dietary fibre consumption is strikingly low in Hungary which favours the development of the so-called civilization diseases like disorders of the colon, diabetes, hypertension, etc. The present state of dietary fibre consumption in Hungary was the topic of the symposium held in Pécs where experts in medicine, nutrition and food industry have discussed the role of dietary fibre in development of different illnesses as well as its therapeutical effect. The round-table conference of the symposium has determined the main lines of research.

The book may be useful to the specialists dealing with the problems of dietary fibre in the most different fields: medicine, industry and nutritional science.

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